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Page 134, line 18

For 0·5 per cent. sodium nitrite

read 0·5 per cent. potassium ferricyanide

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The Nature of Dormancy in the Tea Plant

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Received 6 April 1954

TEA shoots go through alternating states of growth and dormancy. The term 'flush' has been applied to the amount of growth produced by the terminal bud between two states of dormancy. The number of flushes per annum does not exceed five in north-east India (*Ann. Rep.* 1935). Five flushes are rare and probably confined to particular genotypes. Bond (1942) observed up to four flushes in tea plants growing in Ceylon.

When the bud breaks its dormancy, small prophylls with abbreviated internodes are produced, followed by foliage leaves. It has been observed that particular shoots make flushes at definite times of the year (*Ann. Rep.* 1935).

In north-east India tea bushes are pruned, usually annually, to a flat surface, leaving about half an inch of the last extension growth as an increment to the frame. Almost the entire crop of maiden shoots is produced by the outgrowth of buds on the stub which is left behind at pruning. When these shoots reach a predetermined height (generally about 8 in.) above the pruning level, then they are plucked (decapitated) to form a flat horizontal surface. The decapitation stimulates the axillary buds to produce shoots, the more distal buds receiving the maximum stimulus. The axillary shoots are plucked in their turn, as near as possible to the original level of plucking; and there remains behind a very small piece of stem which carries minute buds in the axils of deciduous prophylls. These buds are stimulated to produce a crop of shoots of the second *order*. The process is repeated until the end of the season. The system attains a complexity such that shoots of many different orders are plucked at the same time.

Shoots which project above the plucking level by two leaves or more are removed (plucked). Shoots with one leaf above the level are plucked at the next 'round', generally 7 days later. There are minor modifications in respect of the number of leaves which determine a pluckable shoot. All systems in north-east India produce a horizontal table of cropped vegetation looking like a raised lawn and similar in nature to a clipped hedge; but, as described, the cropping is slightly selective. It will be clear that plucking very greatly increases the number of shoots.

The growing terminal bud is tightly enclosed by one leaf; this unfolds leaving the bud enclosed by the next outermost leaf. The process of leaf unfolding continues until a much smaller dormant bud, enclosed by prophylls, is eventually disclosed by the unfolding of the ultimate leaf of the flush. The leaves are elliptical, widest about the mid-point of their length (Fig. 1).

Observation of periodic changes in the status of the terminal buds of a

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plucked bush is made difficult by the repeated decapitation of the young shoots; a shoot terminated by an apparently growing bud will generally be plucked before any enclosed prophylls are disclosed to view. If a dormant bud is to grow out as a shoot, then a minimal number of leaves must be unfolded before the next dormant terminal bud can be seen.

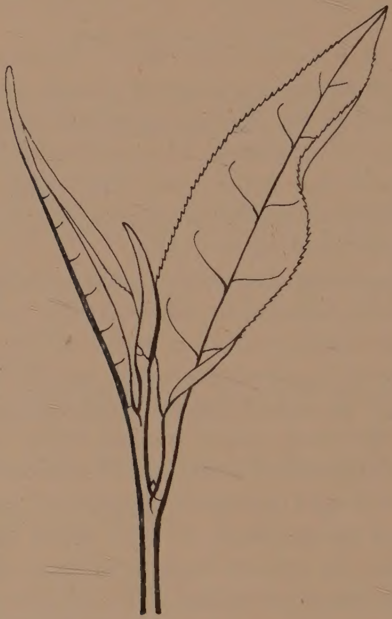

FIG. 1. A tea shoot.

Recurrent states of dormancy of the terminal bud of an elongating tea shoot are due to interruptions in the process of differentiation of leaf initials at the growing apex. A reduced number of leaf initials forecasts the production of a dormant bud. This has been demonstrated by Bond (1945).

Dissection of the buds of plucked shoots shows that when the ratio of the length of the bud to the length of the topmost leaf of the shoot is greater than 0·5, then the number of leaf initials is at least one more than when the ratio is less than 0·5. This is shown in Table I.

TABLE I

No. of leaf initials	Frequency occurrence of buds		Total No. of buds examined
	Ratio $< 0{\cdot}5$	Ratio $> 0{\cdot}5$	
3	2	0	2
4	24	0	24
5	10	26	36
6	0	8	8
3 and 4	26	0	26
5 and 6	10	34	44

χ^2 significant at $P < 0{\cdot}001$

Thus by observing whether the apex of the bud stands above or below the middle of the distal leaf, it is possible to define the morphological status of the bud. That this definition includes the minute topmost internode does not invalidate its use for our purposes.

In 1939 an experimental block of 208 uniformly spaced bushes was divided into two halves. After pruning, the bushes in one half of the block were allowed to grow naturally for the remainder of the season. Under these conditions the shoot population was characterized by strong leading growth; some shoots became dormant after producing one flush, others continued to make a succession of flushes. Soon after pruning, fifteen shoots in a bush were marked at random before their differential nature became apparent. The length of the terminal buds of these shoots, according to whether they were greater or less than half the length of the topmost leaf (in the sense already defined), was determined in the field without removing the shoots from the bushes. The determinations were made at weekly intervals. The frequency occurrence of two classes of shoots was thus obtained and the ratio of those less than a half to those greater than a half was taken as an index of the morphological status of the buds in a bush. The determinations were made visually, without the use of a scale. In practice it was found that the number of buds which appear to be exactly a half is negligible.

In the other half of the experimental block bushes were plucked weekly in the usual way. The total number of plucked shoots was divided into the two classes and an index was calculated in the same way as for the unplucked bushes. Observations of both sets of bushes continued throughout the plucking season.

The indices, obtained on the same dates, for the plucked and the unplucked bushes are plotted against each other in Fig. 2.

The high correlation shows that changes in the morphological condition of the buds in the two sets of bushes proceed simultaneously in the same direction, even though the magnitude of the index is reduced in the one set of bushes due to repeated regeneration under the influence of plucking. The condition of the buds is therefore presumed to be caused by factors inherent in the proximal parts of the plant which remain behind and are not plucked away.

The ratio of the frequencies of the buds less than a half to those greater than a half of the topmost leaf measures an innate growth potential upon which the morphological expression of dormancy is dependent; and we define the ratio as the *dormancy index* of the tea plant. It is to be noted that the method is rapid and may be applied by non-technical personnel in the field.

We found (*Ann. Rep.* 1933) that the water content of the stem is reduced immediately below a dormant terminal bud; and that the water content rises again before the bud starts flushing.

Bond (1945) observed a reduction of the vascular tissue below the growing-point of rapidly elongating periodic tea shoots and concluded that this would restrict the supply of nutrients to the growing apex. The break in the regular sequence of leaf initials which expresses itself as a dormant bud was considered to be caused by the restricted supply of nutrients. Whilst this may be the immediate cause of a dormant bud, yet the data in Fig. 2 suggest that a more remote cause has to be considered.

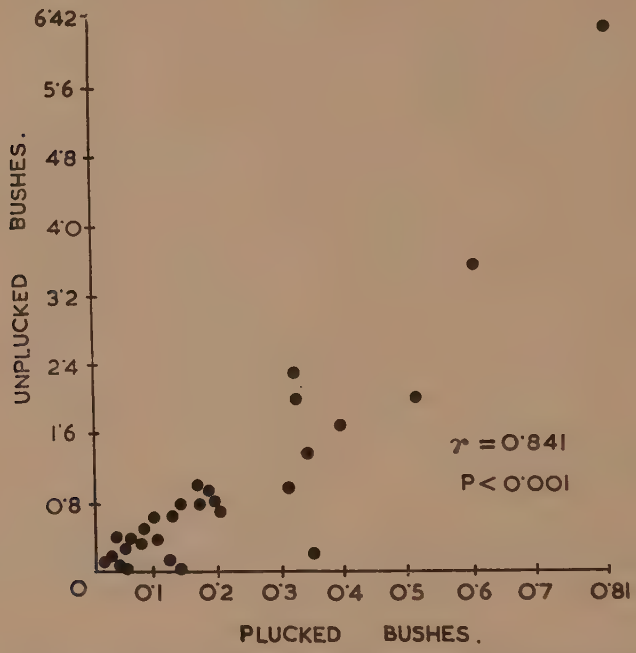

FIG. 2. Relation between the dormancy indices of unplucked and plucked bushes.

Unpublished data in this laboratory show that growth in the length of the feeder root system exhibits a periodic fluctuation similar to that of the top parts of a tea bush. This may be taken as indicative of a periodic rise and fall in the uptake of nutrients by the plant. Equally with a reduction of the vascular tissue below the bud, the periodic growth of the roots might be considered to be the controlling factor in the periodicity of top growth. If the root growth be considered dependent upon a shoot growth which is controlled by a 'bottleneck' in the vascular tissue, then one must acknowledge that the 'bottleneck' has the power of re-establishing itself in newly regenerated shoots in sympathy with that existing in the permanent leaders of a separate plant.

The length of the terminal bud of a tea shoot measures the length of a phyllome; thus, the length of the terminal bud relative to the length of the topmost leaf is a measure of an axial gradient of growth along a caulome. The dormancy index therefore expresses the proportion of shoots in which the gradient is above or below a certain magnitude. Comparison of plucked and unplucked bushes in respect of dormancy index shows that changes in the morphological status of the population of shoots in a bush takes place at a fixed time. One must suppose the change to be dependent upon conditions inherent in the plant and to show an appreciable degree of independence either of the number of shoots or their position of origin in the branch system.

The authors thank the Director of the Tocklai Experimental Station and the Indian Tea Association for permission to publish these results.

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Absorption and Assimilation of Inorganic Nitrogen from Different Sources by Storage Root-Tissue

E. D. H. EL-SHISHINY

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Received 29 March 1954

SUMMARY

1. The rate of ammonium and nitrate absorption and assimilation by storage tissues of sweet potato tubers, radish, and carrot roots cultured in aerated dilute solutions (5 mg. eq./l.) of NH_4Cl, KNO_3, or NH_4NO_3 at 25° C. and the effect of the source of nitrogen on the nitrogenous composition of the tissues were studied.

2. Both ions were absorbed at more or less equal rates under the prevailing experimental conditions by cells of the different plant materials used.

3. The rate of nitrate assimilation was lower than absorption, leading to the accumulation of nitrate-N in the cells of all tissues examined. Ammonium-N, on the other hand, was assimilated almost as soon as it entered the cells of sweet potato tubers and carrot roots, and there was only a small increase in the level of ammonium-N; but in radish root cells ammonium assimilation lagged very much behind absorption, resulting in a great increase in the level of ammonium-N.

4. Sweet potato tubers and carrot tissues were shown to be adapted to the utilization of more ammonium than nitrate nitrogen, but radish root tissues are adapted to the utilization of more nitrate nitrogen.

5. Ammonium or nitrate-N caused similar increases in the complex organic-N level (protein- + rest-N) in radish root, but the rate of synthesis was higher with ammonium in sweet potato tubers and with nitrate in carrot roots.

Furthermore, with both sources changes in the content of amide and amino-N were negligible in radish tissues of both series. In contrast to radish, carrot tissues showed marked increases in the two fractions. The increase of these fractions in sweet potato tuber tissues was much lower.

The marked contrast in nitrogenous composition in the three different plant materials fed with ammonium and nitrate salts separately indicated that the actual course of protein metabolism is most probably dependent on the source of nitrogen utilized.

INTRODUCTION

MOST plant tissues are able to take in and utilize both forms of inorganic nitrogen: ammonium and nitrate. It was generally believed that ammonium-N would be more readily utilized as a source of nitrogen for protein synthesis and that nitrates would first undergo reduction. Thus Prianischnikow (cited by Pardo, 1935) suggested that the nitrate absorbed is reduced to ammonia within the plant as the first step towards the formation of amino acids. Eckerson (1924 and 1932) and Warburg and Negelein (1920) confirmed the

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reduction of nitrate via nitrite and ammonia, and Chibnall (1939) and Virtanen and Arhimo (1939) further suggested the reduction of nitrate via nitrite, hyponitrous acid, hydroxylamine, and ammonia by higher plant tissues.

More recent work has shown that some plants are better adapted to the utilization of oxidized and some to reduced nitrogen. Young rice plants, *Pandanus veichii*, pineapple roots, and potato sprouts show better absorption and assimilation of ammonium than nitrate-N (Bonner, 1946; Sideris, Krauss, and Young, 1937 and 1938; Street, Kenyon, and Watson, 1946). On the other hand, barley, oats, rye, other cereals, and radish root slices show a better utilization of nitrate than ammonium-N (Arnon, 1937; Arenz, 1941; Kappen and Weinhues, 1942; Burström, 1943; Saïd and El-Shishiny, 1949. In other cases more or less equal rates of absorption of both ions from dilute, well-aerated solutions have been reported (Arnon, 1939; Stewart, Thomas, and Horner, 1925; Street, Kenyon, and Watson, 1946).

Evidence confirming the suggestions of Virtanen and Arhimo (1939) that in higher plants the actual course of protein synthesis may vary somewhat according to the nitrogen source utilized were obtained by a number of workers (e.g. Sideris, Krauss, and Young, 1937, 1938; Sideris and Young, 1946; Street, Kenyon, and Watson, 1946).

For a comprehensive and thorough survey of the literature of nitrogen nutrition the reader is referred to recent reviews by Burström (1945), McKee (1937 and 1949), Nightingale (1937 and 1948), and Stewart and Street (1947).

MATERIALS AND METHODS

The slices used in this investigation were taken from tap roots of radish, variety 'Aegyptiacus'; carrot variety 'Chantenay'; and sweet potato variety 'Balady' tubers. Long cylinders, 25 mm. in diameter, were obtained by means of a cork borer. The cylinders were sliced into disks of 1-mm. thickness. The disks were dried by gentle pressure between blotting-paper and then thoroughly mixed. The required samples were prepared from the stock by taking 40 disks at random (20 g.) for each sample. The samples were then placed in net bags in a jar containing 15 l. of sterilized water and aerated by passing a strong current of air through the water for 48 hours for radish and potato and 96 hours for carrot to allow the after effects of wounding to diminish (Saïd and El-Shishiny, 1947). At the end of the washing period the samples were drained and washed several times with distilled water. Two samples were extracted immediately with hot water and analysed for the various nitrogenous fractions, and the remaining samples were transferred to culture vessels containing either 400 ml. distilled water or the experimental solution according to the following scheme: sample 1 and 2: distilled water; sample 3 and 4: 5 m.equiv./l. ammonium chloride; sample 5 and 6: 5 m.equiv./l. potassium nitrate; sample 7 and 8: 5 m.equiv./l. ammonium nitrate.

Immediately after transferring each sample into its respective medium the latter was sampled and the culture vessels were then submerged to the neck in a constant temperature water-bath (25° C. ± 0·1° C.). A current of air was forced through a sintered glass disk at the bottom of each chamber at the rate of 4 l. per hour. After 24 hours the tissue samples were drained, washed with distilled water, extracted immediately, and analysed for the various nitrogenous fractions using a technique standardized by the author (in the press). The medium, together with the washings, was made to a convenient volume and kept under toluene for further analysis.

Separation of insoluble- from soluble-N. The disks in each sample were dropped into 50 ml. boiling distilled water, and boiling was then continued for 3 minutes. The killed tissues were pounded thoroughly in the water extract. The fine thin paste was then quantitatively removed into 150-ml. beaker, 1 ml. of 10 per cent. acetic acid was added, the temperature raised quickly to boiling to coagulate the proteins, and then immediately chilled. The suspension was filtered by suction through Whatman filter paper No. 50. The residue, after washing three times with a little distilled water, was dried and used for the determination of the insoluble-N fraction, while the soluble-N fractions were determined in the combined extract and washings.

Estimation of insoluble-N. Micro-Kjeldahl technique as recommended by Pregl was used.

Estimation of total soluble-N. Three-ml. aliquots of the extract were digested by the reduced iron method of Pucher, Leavenworth, and Vickery (1930), adapted to a micro scale to ensure nitrate reduction. The nitrogen content of the digest was determined as described under insoluble-N.

Free ammonia-N. Ammonia was distilled under reduced pressure at 40° C. in a modified micro-Kjeldahl distillation apparatus (El-Shishiny, in the press), under conditions similar to those adopted by Vickery and Pucher and Leavenworth (1935), using 5-ml. aliquots of the extract. The ammonia was absorbed in 5 ml. N/70 HCl and the excess acid titrated against N/100 NaOH, using Tashiro's indicator.

Nitrate-N. The reduced iron method of Vickery and Pucher (1929) adapted to micro-scale was used. A blank or comparison run must be made without the iron. The difference in the amount of ammonia-N obtained in the two determinations represents the nitrate-N of the sample.

Total amide-N. Aliquots of 5 ml. of the extract were hydrolysed with $2N.H_2SO_4$ in a boiling water bath for 4 hours. The hydrolysate was neutralized to pH 4 with 40 per cent. NaOH, using congo red paper. The ammonia was determined as described for free ammonia-N, for which allowance should be made when calculating the amide-N.

Glutamine amide-N. The mild hydrolysis procedure of Vickery, Pucher, Clark, Chibnall, and Westall (1935) was used.

Amino-N. The method of Pope and Stevens (1939) was used.

EXPERIMENTAL RESULTS

Expt. I. *Absorption and assimilation of inorganic-N from different sources by radish root slices.* The means of duplicate values of ammonium- and nitrate-N absorbed, found in tissues and assimilated by radish root slices cultured in 5 m.equiv./l. of ammonium chloride, potassium nitrate, and ammonium nitrate are presented in Table I, calculated as mg. nitrogen per 100 g. fresh weight of tissue. The assimilated inorganic nitrogen has also been calculated as a percentage of the total available in the tissues, i.e. entering plus initially present.

TABLE I

Radish root. Ammonium and nitrate nitrogen absorbed and assimilated in 24 hours from dilute solutions. Mg. nitrogen (means of duplicate samples) per 100 g. fresh weight of tissue. The amounts assimilated are also given as percentages of the total amount available in the tissues

Culture medium	Absorbed		Available		Found		Assimilation			
	NH_4—N	NO_3—N	NH_4—N	NO_3—N	NH_4—N	NO_3—N	NH_4—N		NO_3—N	
							mg.	%	mg.	%
Distilled water	0·0	0·0	2·16	21·23	1·70	18·69	0·46	21·3	2·54	12·0
5 mg.-equiv./l. NH_4Cl	46·52	0·0	48·68	21·23	36·88	14·46	11·80	24·2	6·77	31·9
5 mg.-equiv./l. KNO_3	0·0	44·99	2·16	66·22	1·52	48·15	0·64	29·6	18·07	27·3
5 mg.-equiv./l. NH_4NO_3	41·36	45·26	43·52	66·49	36·73	42·42	6·79	15·6	24·07	36·2

It appears from Table I that both ammonium and nitrate ions were absorbed at more or less equal rates from dilute, well-aerated solutions of ammonium and nitrate salts. These results are in agreement with previous findings by Arnon (1939), Stewart, Thomas, and Horner (1925), and Street, Kenyon, and Watson (1946), using other species.

The rate of assimilation of both nitrate and ammonium was much lower than the rate of absorption. As much as 73 per cent. of the nitrate remained unchanged in tissues cultured in potassium nitrate solution, and in ammonium chloride solution 76 per cent. of the ammonium-N accumulated in the cells as such. The level of nitrate-N was therefore increased in tissues fed with nitrate solutions as was to be expected from the results obtained by many researchers, but ammonium-N, which usually does not accumulate but is immediately utilized as soon as it is absorbed, also reached, in radish root tissues, levels as high as 50 per cent. of the total soluble nitrogen and 25 per cent. of the total-N. It has been previously found (Saïd and El-Shishiny, 1949) that the presence of high ammonium levels increased the assimilation of nitrate in radish root tissues. This can again be demonstrated by comparing the assimilation of available nitrate by tissues fed with ammonium chloride with that of control tissues in water: 31·9 as against 12 per cent. Compare also 36·2 per cent. in ammonium nitrate with 27·3 per cent. in potassium nitrate.

Radish root tissues appear to be more adapted to nitrate than to ammonium assimilation. Note that in tissues fed with ammonium nitrate the ratio of nitrate to ammonium assimilation was 24·07 : 6·79, i.e. about 3·5. It was interesting to notice that there was no increase in the level of free ammonia-N accompanying nitrate assimilation in tissues fed with potassium nitrate.

Table II gives data for the initial and final distribution of the various forms of nitrogen. No change is shown in the amide-N content in dilute potassium nitrate solution and only a slight increase in ammonium chloride or ammonium nitrate solutions, despite the great increase in the free ammonia-N in both cases.

TABLE II

Radish root. Initial and final distribution of nitrogen in different fractions after 24 hours in distilled water and in solutions containing NO_3— *and/or* NH_4—N. (*mg.* N *per 100 g. fresh weight of tissues—means of duplicate samples*)

Treatment	Nitrate-N	Ammonium-N	Amide-N	Amino-N	Rest-N	Total Sol.-N	Insol.-N	Total-N
Initial values	21·23	2·16	3·05	5·60	16·58	48·62	54·88	103·50
Distilled water	18·69	1·70	3·23	5·91	11·71	41·24	61·55	102·79
5 mg.-equiv./l. NH_4Cl	14·46	36·88	4·74	6·96	10·52	73·56	76·01	149·57
5 mg.-equiv./l. KNO_3	48·15	1·52	3·59	7·32	15·17	75·75	75·33	151·08
5 mg.-equiv./l. NH_4NO_3	42·42	36·73	4·94	7·67	17·19	108·95	83·32	192·27

The original level of amino-N in radish root slices was very low, about 7 per cent. of the total organic-N, and this fraction of the soluble organic-N increased only slightly as a result of culturing in the different solutions.

The inorganic nitrogen assimilated was mainly recovered as protein-N, and in tissues fed with nitrates, in part also as rest-N. The increase of protein-N in the ammonium chloride series amounted to 14·46 mg., which corresponds to 23·5 per cent. of the protein content of the control tissue starved in plain water. The corresponding increases in the potassium nitrate and ammonium nitrate series were 22·4 per cent. and 35·4 per cent.

Expt. II. *Absorption and assimilation of inorganic-N from different sources by slices of sweet potato tuber.* The means of duplicate values of absorbed, found in the tissues and assimilated inorganic-N for slices of sweet potato tubers, obtained as for radish root in Expt. I, are presented in Table III.

TABLE III

Sweet potato tuber. Ammonium and nitrate nitrogen absorbed and assimilated in 24 hours from dilute solutions. Mg. nitrogen (*means of duplicate samples*) *per 100 g. fresh weight of tissue. The amounts assimilated are also given as percentages of the total amounts available in the tissues, i.e. initially present plus absorbed.*

Culture medium	Absorbed		Available		Found		Assimilation			
	NH_4—N	NO_3—N	NH_4—N	NO_3—N	NH_4—N	NO_3—N	NH_4—N		NO_3—N	
							mg.	%	mg.	%
Distilled water	0·0	0·0	0·648	0·0	0·831	0·0	−0·183	—	0·0	0·0
5 mg.-equiv./l. NH_4Cl	69·65	0·0	70·30	0·0	1·88	0·0	68·42	97·3	0·0	0·0
5 mg.-equiv./l. KNO_3	0·0	72·68	0·648	72·68	1·24	26·58	−0·592	—	48·10	66·2
5 mg.-equiv./l. NH_4NO_3	40·25	37·61	40·90	37·61	4·58	12·65	36·32	88·8	24·96	66·4

The results showed that, as with radish root, ammonium- and nitrate-N were taken up at approximately equal rates. The amounts of nitrogen absorbed in 24 hours were 69·65 and 72·68 mg. from ammonium chloride and potassium nitrate solutions respectively, and 40·25 and 37·61 mg. ammonium- and nitrate-N from ammonium nitrate solutions.

The rate of ammonium assimilation exceeded greatly the rate of nitrate assimilation since in ammonium chloride solutions 97·3 per cent. of the available ammonium-N (initially present plus absorbed) was assimilated as compared with 66·2 per cent. of the available nitrate-N in potassium nitrate solution. In ammonium nitrate the rate of ammonium assimilation was slightly below that in ammonium chloride, being 88·8 per cent. of the available ammonium-N in the tissues. These results indicate that ammonium ion was very rapidly metabolized and did not accumulate in appreciable amounts. Nitrate feeding, on the other hand, was associated with pronounced accumulation of nitrate-N, since as much as about 34 per cent. of the nitrate taken in from potassium or ammonium nitrate culture media remained as such in the tissues despite the high carbohydrate level.

TABLE IV

Sweet potato tuber. Initial and final distribution of nitrogen in different fractions after 24 hours in distilled water and in solutions containing NO_3— *and/or* NH_4—N (*mg. nitrogen per 100 g. fresh weight of tissues—means of duplicate samples*)

			Amide-N						
Treatment	Nitrate-N	Ammonium-N	glutamine	Asparagine	Amino-N	Rest-N	Total Sol.-N	Insol.-N	Total-N
Initial values	0·0	0·648	—	—	—	—	—	—	—
Distilled water	0·0	0·831	1·60	1·33	33·67	22·96	60·48	74·38	134·86
5 mg.-equiv./l. NH_4Cl	0·0	1·88	6·47	4·53	39·40	55·12	107·40	90·56	197·96
5 mg.-equiv./l. KNO_3	26·58	1·24	2·46	1·23	48·86	51·86	132·23	80·38	212·61
5 mg.-equiv./l. NH_4NO_3	12·65	4·58	6·95	3·34	43·12	49·45	120·09	86·31	206·40

The initial and final distribution of the different nitrogenous fractions are recorded in Table IV. In ammonium chloride the ammonium-N that disappeared from the external medium was recovered as increases in amide-, amino-, rest-, and protein-N fractions. There was an insignificant increase in the amide-N content of tissues in potassium nitrate, but significant increases were recorded in amino-, rest-, and protein-N. Contrary to what was expected from our experience with radish and carrot root tissues, the greatest increase in complex organic-N—rest-N and protein-N fractions—was recorded in tissues cultured in ammonium chloride (32·16 mg. rest-N and 16·18 mg. protein-N) and not in potassium nitrate (28·9 mg. rest-N, and only 6·0 mg. protein-N). The increase of organic-N in the tissues of the nitrate series was mainly due to increase in the amino-N and rest-N fractions which corresponds to 86·6 per cent. of the assimilated inorganic-N. A considerable increase in amide-N of 8·07 mg., 4·87 mg. due to glutamine and 3·2 mg. due to asparagine, was recorded in tissues cultured in ammonium chloride.

Expt. III.[1] *Absorption and assimilation of inorganic-N from different sources by carrot root slices.* Data for carrot corresponding to those obtained in Expts. I and II in ammonium chloride and potassium nitrate solutions are presented in Tables V and VI.

TABLE V

Carrot root. Ammonium and nitrate nitrogen absorbed and assimilated in 24 hours from dilute solutions. Mg. nitrogen (means of duplicate samples) per 100 g. fresh weight of tissue. The amounts assimilated are also given as percentages of the total amount available in the tissues, i.e. initially present plus absorbed

Culture medium	Absorbed		Available		Found		Assimilated			
	NH_4—N	NO_3—N	NH_4—N	NO_3—N	NH_4—N	NO_3—N	NH_4—N		NO_3—N	
							mg.	%	mg.	%
Distilled water	0·0	0·0	0·46	0·0	0·31	0·0	0·15	—	0·0	0·0
5 mg.-equiv./l. NH_4Cl	53·23	0·0	53·69	0·0	9·95	0·0	43·74	81·5	0·0	0·0
5 mg.-equiv./l. KNO_3	0·0	50·96	0·46	50·96	0·78	23·16	−0·32	—	27·80	54·55

TABLE VI

Carrot root. Initial and final distribution of nitrogenous fractions after 24 hours in distilled water and in solutions containing NO_3 *or* NH_4—N *(mg. nitrogen per 100 fresh weight of tissue means of duplicate samples)*

Treatment	Nitrate-N	Ammonium-N	Amide-N Glutamine	Amide-N Asparagine	Amino-N	Rest-N	Total Sol.-N	Insol.-N	Total N
Initial values	0·0	0·46	1·56	2·17	21·02	11·35	36·56	56·04	92·60
Distilled water	0·0	0·31	0·47	0·95	14·25	29·07	45·05	46·40	91·45
5 mg.-equiv./l. NH_4Cl	0·0	9·95	14·70	2·91	27·96	40·20	95·72	49·22	144·94
5 mg.-equiv./l. KNO_3	23·16	0·78	2·51	2·19	25·43	28·31	82·38	58·74	141·12

Here again the two forms of nitrogen were taken in at about the same rate, 53·2 mg. ammonium-N and 50·96 mg. nitrate-N being removed from ammonium chloride and potassium nitrate solutions respectively in 24 hours. Unlike radish root, carrot root tissues appeared to be more adapted to the utilization of ammonium than nitrate. They assimilated about 82 per cent. of the available ammonium-N in comparison with only about 55 per cent. of the corresponding nitrate-N. The rate of intake of ammonium and nitrate-N was higher than that of assimilation, but the difference was much greater with the latter, leading to the accumulation of more nitrate than ammonium. Reduction of nitrate-N was not accompanied by a significant increase in the ammonium-N level in carrot root disks, a result which is hardly in favour of the classical view of nitrate reduction to ammonia prior to its transformation into organic-N.

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[1] This experiment is part of one of the experiments carried out, jointly with Mr. M. A. Noseir, to show the effect of some antibiotics on the nitrogen metabolism of storage root tissues. The complete results of these experiments will be the subject of a subsequent paper.

The data recorded in Table VI show a greater increase in total organic-N in the ammonium than in the nitrate series. The increase was mainly due to the soluble forms in ammonium chloride, 16·19 mg. amide-, 13·71 amino-, 11·13 mg. rest-N, but only 2·82 mg. protein-N above the corresponding levels of the control disks in distilled water. On the other hand, in potassium nitrate the increase in organic-N was mainly due to amino- and protein-N: 11·18 mg. of the former and 12·34 mg. of the latter. The amide-N level remained relatively low with an increase of only 3·28 mg.

It appears from these results that carrot root disks form more protein with nitrate than with ammonium, and that ammonium assimilation in carrot roots is correlated with increases in all the soluble nitrogenous compounds. On a percentage basis the increases in protein and amino-N fractions in the nitrate series correspond to about 47·4 per cent. and 42·9 per cent., respectively, of the assimilated nitrate-N, while the increase in the amide-N corresponds to only 12·6 per cent. On the other hand, the increases in amide-, amino-, and rest-N correspond to 36·9, 31·3, and 25·4 per cent. of the assimilated ammonia, while only 6·4 per cent. appeared as increase in protein. This marked contrast in the composition of ammonium and nitrate cultured tissues supports the suggestion that the actual course of protein synthesis may be somewhat different according to the nitrogen source utilized (Sideris *et al.*, 1937, 1938, 1946; Street *et al.*, 1946).

The increase in the amide-N fraction in the ammonium-fed series which amounted to 16·19 mg., was mainly glutamine, the asparagine contributing only 1·96 mg. The increase in total amide-N in the nitrate series was 3·28 mg. only, of which 1·24 mg. were due to asparagine.

DISCUSSION

When the rates of absorption and assimilation of ammonium- and nitrate-N by different plant materials, and also the distribution of the products of assimilation of inorganic-N among the various forms of organic nitrogenous compounds, provided by the work of many investigators, are compared, it appears that different plants may vary with respect to the details of their metabolic processes.

Despite the fact that all plants may possess the ability to utilize both forms of inorganic nitrogen, ammonium, and nitrate, yet certain plants appear to make use of one form rather than the other. Radish root disks in the present experiments utilized more nitrate than ammonium, although both ions were absorbed at more or less equal rates from dilute aerated solutions of ammonium chloride; potassium nitrate and ammonium nitrate, yet the rate of nitrate-N assimilation exceeded that of ammonium. In ammonium nitrate, where both ions were present at the same time, the rate of nitrate assimilation was about 4 times that of ammonium: on a percentage basis, 36·2 per cent. of the nitrate-N as compared with 15·6 per cent. of the ammonium-N was transformed into organic-N. These results place radish roots in that group of nitrate plants, like certain cereals, adapted to utilize nitrate more readily than ammonium-N, as recorded by Arenz (1941), Arnon (1937), Burström (1943), and Kappen and Weinhues (1942).

On the other hand, the storage tissues of carrot roots and sweet potato tubers behaved differently. In respect of their ability to utilize ammonia more readily than nitrate, they are similar to rice plants, roots of *Pandanus veichii* and *Ananas comosus*, and potato sprouts as reported by Bonner (1946), Sideris, Krauss, and Young (1937 and 1938), and Street, Kenyon, and Watson (1946). In both carrot root and sweet potato tuber, nitrate was not assimilated as readily as ammonium although the oxygen supply and carbohydrates were plentiful. This may indicate that the slow rate of nitrate assimilation is due to inherent causes. A similar result was obtained for Pandanus roots by Sideris, Krauss, and Young (1937).

The rate of nitrate assimilation in the storage tissues of all three plant materials used in the present experiments was below the rate of nitrate absorption and therefore nitrate feeding led always to pronounced accumulation of nitrate-N in the tissues, a result which has been repeatedly recorded by other investigators (e.g. Nightingale, 1937).

The assimilation of nitrate-N, as appeared from the results recorded in Tables II, IV, and VI, for tissues cultured in dilute potassium nitrate solutions, did not lead to any significant increase in the ammonium or amide-N levels in the tissues. This result may indicate that nitrate was not reduced in the cells into ammonia or that the rate of ammonium transformation into organic-N other than amide-N, is as fast as its production from the nitrates.

The ammonium-N absorbed by sweet potato tuber and carrot root tissues was very rapidly metabolized and did not accumulate in any appreciable amounts in the cells.

Radish root tissues behaved differently in that the rate of ammonium assimilation is far below the rate of absorption. The level of ammonium-N rose to 50 per cent. and 34 per cent., respectively, of the total soluble nitrogen. There were no symptoms of toxicity nor significant stimulation of amide synthesis, a result which is similar to those of Vickery *et al.* (1938–40), who reported that rhubarb and tobacco tissues could accumulate and withstand high levels of ammonium-N without showing any symptoms of toxicity, thus again invalidating the 'detoxication' hypothesis and discrediting the stimulating effect of ammonia in amide synthesis.

In a comparative study of the distribution of the assimilated inorganic-N among the various forms of soluble and insoluble organic nitrogenous fractions in the ammonium and nitrate series, it appeared that the mode of assimilation of the ammonium-N was quite different from that of the nitrate-N.

The increase in the total organic-N was greater in the ammonium series for carrot and sweet potato, where ammonium was more readily assimilated than nitrate, but in radish the reverse was the case.

Results of other workers have indicated that nitrate feeding is usually associated with a comparatively high content of complex organic forms of nitrogen in contrast to ammonium feeding, which is usually associated with accumulation of amino-acids and amides. Radish proves to be different: here the increase in the complex organic-N, when calculated as a percentage of the total increase in the organic-N, is about the same in ammonium chloride as in potassium nitrate. In sweet potato more of the complex organic nitrogenous compounds were formed with ammonium than with nitrate feeding: the percentage increase of protein-N in the ammonium series was more than twice that in the nitrate series. In carrot root the increase in the complex organic-N (rest-N+protein-N) was less than 50 per cent. of the increase in total organic-N in ammonium chloride or potassium nitrate. The storage tissues of carrot roots thus behaved differently from those of radish root and sweet potato tubers, since the newly formed protein-N with nitrate feeding was 47·4 per cent. in contrast to 6·3 per cent. of the total increase in organic-N with ammonium feeding. Carrot root tissues behave in this respect like *Pandanus veichii* and *Ananas comosus* roots as reported by Sideris, Krauss, and Young (1937 and 1938). On the other hand, more protein-N was formed with ammonium than with nitrate in radish roots and sweet potato tubers, which behaved in this respect like tomato plants as reported by Clark (1936).

The results of these experiments place the sweet potato tubers in the group of plants which contain both glutamine and asparagine and are capable of synthetizing both amides, and place carrot roots in that group which accumulate glutamine only, when placed under conditions leading to an increase in the ammonium-N level in the tissues. They also place radish root among the group of plants like tobacco (Vickery *et al.*, 1940) in which amide synthesis is but slightly stimulated by increasing the concentration of ammonia in the cells.

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The Significance of the Measurement made by the Cobalt Chloride Paper Method

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A RECENT article (Bailey *et al.* 1952) reflects the general lack of appreciation of what is really measured by Stahl's cobalt chloride paper method. Although many of the theoretical shortcomings of the technique are recognized, it is still regarded as a method for measuring the *transpiration rate*, whereas it more truly estimates the *diffusive conductance of the stomata*. That this is so follows from a simple consideration of the vapour phase of the transpiration stream. The transpiration rate (T) may be represented by

$$T = k(e_s - e_a)f(d)\phi(S)\psi(L),$$

where k is the diffusion coefficient of water vapour in air; e_s the vapour pressure at the evaporating surface within the leaf and e_a that of the ambient air; d the boundary layer of stagnant air overlying the leaf and through which vapour has to diffuse; S the diffusive conductance of the stomata; and L various leaf conductances (or impedances) including that of the cuticle and those in the path of the vapour movement between the evaporating surface and the stomata.

Of the three functions, $f(d)$, $\phi(S)$, and $\psi(L)$, reliable numerical estimates are available only for the first. The magnitude of the boundary layer is determined mainly by the wind velocity and surface dimensions, and for small isolated surfaces such as single leaves, $f(d)$ may be taken approximately as $Cl^{0\cdot7}b^{0\cdot8}\mu^{0\cdot5}$ (Martin, 1943) or more exactly as $Cl^{0\cdot73}b^{0\cdot8}(1+0\cdot121\mu^{0\cdot85})$ (Powell and Griffiths, 1935), where l and b are respectively the surface dimensions parallel and normal to the windstream of velocity μ, and C is a proportionality constant. In a field with a continuous cover of vegetation, the boundary layer may be assumed to be similar to that of an open water surface, and thus equals $C(1+0\cdot01\mu)$ (Penman, 1948). No empirical estimations of $\phi(S)$ have yet been made. Nevertheless, with open stomata and the leaf ventilated, the resistance of the stomata to diffusion is likely to be less than half of that imposed by d (Penman and Schofield, 1952). Impedances to vapour movement within the leaf include the cuticle, the length of the mean diffusion path between the cell wall and the stomata, and the resistance imposed by the interstices of the cell walls through which vapour moves from the evaporating surface located within to the edge of the intercellular spaces. All are likely to be small and constant over short periods of time for a leaf of any

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one species which has always had an adequate water supply, but they will probably vary between species.

When a piece of dry paper, as in Stahl's method, is clamped over a leaf, the transpiring system is immediately subjected to two important changes. First, the boundary layer is almost entirely removed, as the absorbing surface instead of being, say, some 2 mm. distant, is now close to the mouths of the stomata. (Just how close will depend on the hairiness of the surface.) That is, the effective length of the diffusion path may be reduced by as much as two-thirds (depending on stomatal aperture and wind velocity for any one species). If the usual 'quantitative' method, as used by Bailey *et al.*, is adopted, this factor will tend always to give a higher apparent as compared with actual transpiration rate. Moreover, as the boundary layer is variable and is an important component governing transpiration, large discrepancies, even in qualitative estimations, would certainly be expected. Secondly, the vapour phase at both source (e_s) and sink (e_a), and consequently the concentration gradient, is altered. A small change in e_s may be expected due to a local change in temperature of the enclosed area, despite conduction along the leaf. In the cobalt chloride paper-leaf system, e_a varies during each determination from a very low value when the paper is first applied up to a constant (unknown) level. The integrated mean may therefore be taken as constant. With a transpiring leaf, although e_a remains relatively constant throughout any one clear day, it does vary considerably between different days. The interference with the vapour-pressure gradient, then, would work in the direction of giving reasonably parallel (but not coincident) results between cobalt chloride paper determinations and the true transpiration rate on any one day with much greater variations between different days.

The above considerations alone show that this method is valueless as a field method for measuring the transpiration rate. As a hypothetical example, one may imagine the same leaf at two different times with the same leaf temperature and same stomatal aperture but subjected to a wind of different velocity. The cobalt chloride paper result will be the same in both cases, but the two transpiration rates will be quite different. In the greenhouse, where boundary-layer conditions are more constant, the method is likely to give a closer qualitative estimate than in the field.

The two main factors which render this method inapplicable for measuring transpiration, i.e. removal of the boundary layer and imposition of constant 'sink' strength of vapour pressure, make it an approximate method of determining stomatal conductance to diffusive flow. In the system there are only two important variables: the vapour pressure of the evaporating surface and the stomatal aperture. The former may be approximately calculated from thermocouple measurements and suitably adjusted.[1] Internal resistances, including that of the cuticle, are likely to be relatively constant for any one species. Stahl's method, therefore, offers considerable promise as a field method for estimating stomatal conductance if calibrated against a more exact method; in fact, as used by Livingston and Shreve (1916) and Meyer (1927), this method is no doubt the simplest and most exact qualitative field technique yet devised for measuring stomatal conductance, albeit so elaborately termed the 'index of foliar transpiring power' or 'standard rate of water-vapour loss'. Two important inherent disadvantages of the method remain: (1) if the paper remains on the leaf for more than about 2 minutes, the stomata in the experimental area are likely to change in aperture from those elsewhere on the leaf; and (2) it is subject to the errors of all methods which depend on the human eye to match colour standards.

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[1] It is important to realize that adjustment to a constant vapour pressure gradient from temperature measurements, while giving a more exact estimate of the stomatal conductance, provides a less correct measure of the transpiration rate.

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Interactions of Nitrogen, Phosphorus, and Potassium supplied in Leaf Sprays or in Fertilizer added to the Soil[1]

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SUMMARY

When sugar-beet plants grown in pots were sprayed daily with nutrient solutions supplying nitrogen, phosphorus, and potassium separately or in all combinations, with precautions to prevent spray falling on the soil in which the plants were grown, all three nutrients were absorbed through the leaves. In one experiment nitrogen and potassium, and in another only nitrogen, caused increases in plant dry weight and leaf area. Swedes absorbed phosphorus from leaf sprays and from fertilizer applied to the soil, but only the fertilizer caused an increase in dry weight.

Absorption of any of the nutrients tested from a spray containing more than one nutrient was unaffected by the presence of others in the spray, but spraying with nitrogen-containing solutions increased the absorption of phosphorus and potassium from the soil, and potassium in sprays increased the uptake of phosphorus from the soil.

Nitrogenous fertilizer applied to the soil increased the leaf area of sugar-beet plants, and hence it also increased the amounts of nitrogen, phosphorus, and potassium deposited on the leaves when they were sprayed with solutions of these nutrients, and the amounts absorbed from the spray into the plants. Phosphatic fertilizer had no effect on uptake from leaf sprays. Potassic fertilizer did not affect leaf area or the estimated volume of spray solution retained on the leaves, but it appeared to reduce uptake of potassium from the spray.

Dry weight per plant was increased by all three nutrients in fertilizer, and sugar yield of the roots was increased by nitrogen and potassium in fertilizer, and by nitrogen in spray. Application of a nutrient in leaf spray reduced the responses in dry weight and sugar yield to the same nutrient applied in fertilizer to the soil.

Less nitrogen, but more phosphorus, was taken up from the leaf sprays than from fertilizer. Nutrients from sprays produced smaller increases in total dry weight and in dry weight per unit of absorbed nutrient than the same nutrient from fertilizer.

The apparent percentage recovery of nitrogen applied in spray, based on estimates of the volumes of solution retained on the leaves, was unaffected by fertilizer treatment, that of phosphorus was increased by nitrogen fertilizer, and that of potassium was increased by nitrogen fertilizer and reduced by potassium fertilizer. The volume of spray solution held on the leaves was probably overestimated, so that the highest apparent recovery, about 60 per cent., may

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represent an almost complete true recovery, because only trivial amounts of the nutrients that had been applied in spray remained on the leaf surface to be removed by washing before harvest. Lower apparent recoveries may be due to reduced uptake from the soil of the nutrient supplied in spray.

INTRODUCTION

EXPERIMENTS previously reported (Thorne, 1954) showed that when the leaves of plants of several economic species were sprayed daily with nutrient solutions supplying nitrogen, phosphorus, and potassium, with precautions to prevent spray falling on the soil in which the plants were grown, the nutrient content and dry weight per plant were increased compared with those of control plants sprayed only with water. These results established that nutrients were absorbed by the leaves, but the difference between sprayed and unsprayed plants in the content of a particular nutrient, A, was not necessarily an accurate measure of the amount of A absorbed by the leaves, because uptake of another nutrient, B, from the spray might have caused increased absorption of A by the roots. For the same reason, increases in nitrogen, phosphorus, and potassium content of plants sprayed with a solution containing all three nutrients did not necessarily imply that all three were absorbed through the leaves, but increase in the content of a particular nutrient per cent. of dry matter, accompanying an increase in content per plant, was held to indicate that the nutrient had been absorbed by the leaves. On this criterion, the experiments showed that nitrogen and potassium were consistently so absorbed, but the increase in phosphorus content in some experiments may have been due to increased uptake by the roots. In order to distinguish between the effects of individual nutrients, experiments were done in which nutrients in sprays or in fertilizer added to the soil were applied singly or in factorial combination.

The first experiment described in this paper, done in 1951, was designed to investigate the effect of absorption of one nutrient through the leaves on uptake of other nutrients, either by the leaves or by the roots, by comparing sprays supplying all combinations of the presence and absence of nitrogen, phosphorus, and potassium. This experiment showed that nitrogen and potassium were absorbed from the spray and caused increases in plant dry weight, but did not definitely establish that phosphorus was absorbed.

A second experiment was done in 1952 in order: (1) to confirm that nitrogen, phosphorus, and potassium are absorbed by leaves from sprays; (2) to investigate the effect of nitrogen, phosphorus, and potassium supplied to the roots on the response to the same nutrients applied in sprays; (3) to compare the effects of nitrogen, phosphorus, and potassium supplied in sprays with their effects when applied to the soil as fertilizer. Water or solutions supplying nitrogen, phosphorus, or potassium separately, were sprayed on the leaves of sugar-beet plants grown in pots and receiving all combinations of the presence and absence of nitrogen, phosphorus, and potassium as fertilizer added to the soil. An impracticable number of plants would have been required to make the spray as well as the fertilizer treatments factorial. It was also an advantage to apply the spray nutrients singly because higher concentrations of each nutrient than in the first experiment could be used without danger of scorching the leaves, and it was hoped that this would provide definite evidence of uptake of phosphorus by the leaves as well as of nitrogen and potassium. In order to obtain additional evidence on phosphorus uptake by leaves, the phosphorus spray was also tested on swedes (Expt. 3) which are more responsive than sugar-beet to phosphorus.

EXPERIMENTAL TECHNIQUE

Sugar-beet of a non-bolting Kleinwanzleben strain, supplied by the Plant Breeding Institute, Cambridge, was used for Expts. 1 and 2, and swedes, variety 'Wilhelmsburger', were used for Expt. 3. Expt. 1 was sown on 30 March 1951 and Expts. 2 and 3 were sown on 19 April 1952. All plants were grown singly in glazed pots containing 10 kg. of a mixture of Rothamsted soil and 20 per cent. sand. Each pot in Expt. 1 received a dressing of 6 g. $(NH_4)_2SO_4$ and 3·3 g. K_2HPO_4 at sowing. Analysis of the soil-sand mixture used for Expts. 2 and 3 showed it to be very low in nitrogen and fairly low in potash; there was adequate phosphorus for most crops.

The plants of Expt. 1 were sprayed once daily on 6 days a week with solutions containing the 8 factorial combinations of O *v.* N, O *v.* P, and O *v.* K; N was supplied as 0·08 M. NH_4NO_3, P as 0·005 M. $Ca(H_2PO_4)_2.H_2O$, and K as 0·06 M. KCl. The experiment was harvested on 4 occasions, the first on 13 June just before spraying started and the others 4, 8, and 14 weeks later. The 4 replicates of each treatment taken at each time were bulked in pairs for chemical analysis.

The soil treatments for the sugar-beet of Expt. 2 were the 8 factorial combinations of O *v.* N, O *v.* P, O *v.* K. N was supplied as 4·5 g. NH_4NO_3, P as 3·8 g. $Ca(H_2PO_4)_2.H_2O$, and K as 5·0 g. KCl per pot. These fertilizers were mixed with the soil before sowing. Plants receiving each of the fertilizer combinations were sprayed 6 times a week starting on 5 June, with water, 0·25 M. NH_4NO_3, 0·05 M. $Ca(H_2PO_4)_2.H_2O$, or 0·25 M. KCl. The 32 treatments were replicated 3 times and the experiment was harvested on 30 September. The swedes of Expt. 3, which had a basal NK fertilizer dressing, received the three treatments: phosphorus spray, phosphorus fertilizer and water spray, or water spray. The fertilizer and sprays were the same as for Expt. 2. There were 4 replicates of each treatment and the experiment was harvested early, on 24 July, because the growing points of several of the plants had been damaged by dipterous larvae. In all three experiments the control treatment was spraying with water and all sprays contained 0·05 per cent. sulphonated lorol as a spreader.

The methods used for growing and spraying plants and the observations made on them were similar to those already described (Thorne, 1954). Dead leaves (i.e. lamina and petiole material which became senescent during the experiment) were collected from each plant and added to the lamina fraction at harvest in Expt. 1, and in Expts. 2 and 3 weighed and analysed separately from the rest of the plant material. In Expt. 1 the leaf areas of the harvested plants and of those to be harvested on the next occasion were determined at each harvest, and the leaf areas and leaf numbers per plant of all plants of Expt. 2 were measured at fortnightly intervals. Leaf area was estimated by comparing each leaf of the plant with a set of standard leaf shapes, prepared from photographs of sugar-beet leaves, and forming a geometric series of increasing area (cf. Bald, 1943). The total leaf area of the plant was then obtained by adding the areas of the individual leaves. This method is preferable to the one used previously, which involved weighing counted numbers of leaf disks of known area; it is more accurate for small plants and does not involve destroying the plant.

To find out whether spraying the leaves with nutrient solutions affected their efficiency in producing dry matter, the net assimilation rate (NAR) was calculated in Expt. 1 from the leaf area and dry weight data for each sampling interval by the usual method (Williams, 1946). To increase the accuracy of the estimates of dry weight increment for each sampling interval, initial dry weights were corrected to correspond to the initial leaf areas of the plants harvested at the end of the interval, by means of a regression of plant dry weight on leaf area calculated for the plants harvested at the beginning of the interval.

An attempt was made in Expt. 2 to estimate the quantity of nutrients supplied in sprays. Once a fortnight the volume of spray applied to 3 replicate plants was measured separately for each of the 3 nutrient sprays on plants receiving each of the 8 fertilizer treatments. The volume of spray retained by the leaves was less than the measured volume leaving the spray gun, because some spray was retained by the shield which surrounded the sprayed plants and a small amount drifted away as a fine mist. An estimate of the quantity of spray not retained by the leaves was made from the change in weight of blotting-paper which was held round the plants being sprayed. This shield did not catch the finest droplets of spray, and some of the solution retained by it probably evaporated before weighing because the same blotting-paper shield was used for the 3 replicate plants and so was exposed to the air for some time before being weighed in a tin. The difference between the volume of solution leaving the spray gun and the volume retained by the shield was used as an estimate of the quantity of spray retained by the leaves on the day when the measurements were made. The quantity of nutrients deposited on the leaves during the fortnightly period between measurements was determined from the average volume of spray retained, the concentration of the spray, and the frequency of spraying. The amount of phosphorus applied in spray to the swedes of Expt. 3 was estimated in a similar manner.

On the day before harvest all plants of Expt. 1 were washed with a spray of water in order to remove nutrients deposited on the surface of the leaves.

The quantity of nutrients remaining on the outside of the leaves at harvest was estimated in Expts. 2 and 3 by washing each plant with water and analysing the washings. The top of a single plant was washed in 300 ml. of distilled water and wiped with filter paper to ensure that no persistent deposit of salt remained. The leaves were then rinsed by means of a pipette with 100 ml. of clean water and dried. The solutions collected from washing the 3 replicate plants of each treatment were bulked; the debris was removed by filtering on a Buchner funnel and washed with hot water, and the solutions were made up to volume and analysed for nitrogen, phosphorus, and potassium by the same methods as were used for the plant material (Thorne, 1954).

PRESENTATION OF RESULTS

In the following account the term 'fertilizer' is used for nutrients applied to the soil as opposed to those applied to the leaves in sprays. As in the previous paper (Thorne, 1954) the term 'nutrient content' is used for the weight of a nutrient in a single entire plant, and the difference in the mean content of a particular nutrient between plants receiving that nutrient in spray or fertilizer and those not receiving it is called 'uptake from spray or fertilizer'. Total nutrient content per plant was determined from the dry weights and analyses of lamina (including dead leaves), petiole, and roots in Expt. 1, and tops (lamina plus petiole), roots, and dead leaves in Expts. 2 and 3. The dead leaves were not washed before analysis because of their fragile nature. An estimate of the nutrients deposited on the dead leaves, and therefore included in the total nutrient content, was made in Expts. 2 and 3 by assuming that the quantity of nutrient deposited per g. dry weight of leaf material was the same for the dead leaves as for the tops at harvest, which was known from the analyses of the solutions in which the tops were washed. No nitrogen was detected in the washing solution from any of the plants. The estimated quantity of phosphorus remaining as a deposit on the outside of the dead leaves, averaging all fertilizer treatments of the sugar-beet, was 6·2 mg. per plant, or 2·4 per cent. of the total phosphorus content of a sprayed plant. The corresponding figure for potassium was 8·3 mg. or 0·5 per cent. of the mean potassium content of sprayed plants. For the swedes the average amount of phosphorus deposited on the dead leaves was 9·5 mg. per plant, or 6·7 per cent. of the total phosphorus content. This, though large compared with the value for the sugar-beet, was only slightly larger than the standard error of the mean phosphorus content of the sprayed plants (Table VIII). Estimates of the nutrients deposited from the spray on the dead leaves of plants of each fertilizer treatment vary slightly from the means given above, but in all cases the nutrient contents per plant obtained by adding the contents of tops, roots, and unwashed dead leaves, are only slight over-estimates of the true values. In the following results no allowance has been made for this external deposit of nutrients on the dead leaves.

Plants of different fertilizer treatments received different quantities of nutrients from the spray because of the variation in leaf area resulting from the soil treatments. Hence, to obtain a measure of nutrient uptake independent of the differences in leaf area induced by varying nutrient supply, the percentage recoveries in the plant of nutrients applied in spray (i.e. increases in nutrient content per unit nutrient supplied) were calculated in Expts. 2 and 3. The proportion of the nutrients deposited on the leaf which were absorbed by the plant could not be determined precisely because the actual quantity of nutrients retained by the leaf was not known. The apparent percentage recoveries, based on the estimated quantity of nutrients retained by the leaves (p. 23), were used for comparison. These estimates were not replicated so the standard errors of the apparent recoveries cannot be calculated.

RESULTS

Expt. 1. Sugar-beet

There were no significant interactions between nutrients in the spray solutions, that is, the effect of any nutrient was independent of the presence or absence of other nutrients in the spray. Only the mean effects of nitrogen, phosphorus, and potassium therefore need be considered, and these are given in Tables I and II together with means of all treatments to show changes with time. Standard errors are given in the tables for the increases in dry weight, nutrient content, &c., produced at each harvest by nutrients in sprays; the standard errors of the means per plant are half those of the increases. Due to the bulking of the samples for chemical analysis, standard errors for nutrient contents and derived data are based on only 9 degrees of freedom ($t = 2{\cdot}26$, $p = 0{\cdot}05$), but those for dry weight, leaf area, NAR, and sugar content are based on 27 degrees of freedom ($t = 2{\cdot}05$, $p = 0{\cdot}05$).

Leaf area and dry weight (Table I). Leaf area per plant was increased by nitrogen-containing sprays at all harvests above that of plants not receiving nitrogen. Phosphorus had no effect and potassium gave an almost significant increase at the last harvest. Total dry weight per plant was increased by nitrogen at all harvests and by potassium at only the last.

Nutrient content (Table I). The nitrogen content of plants receiving nitrogen-containing sprays was greater at all harvests than that of plants not receiving nitrogen. Phosphorus content was increased by phosphorus in sprays at only the last harvest, but it was also slightly increased by nitrogen and by potassium at the third harvest. Potassium content was increased by potassium in sprays at all harvests and by nitrogen at the third.

These results show that spraying with a particular nutrient, e.g. nitrogen, caused increases in the content of that nutrient, presumably due to absorption from the spray, and also influenced uptake of other nutrients, e.g. phosphorus and potassium, from the soil. Similarly, nutrients applied in sprays affected the contents of sodium and calcium, which were absent from all the spray

TABLE I

The response at intervals during the growing season of sugar-beet plants to nitrogen, phosphorus, or potassium applied in sprays. Mean increase produced by nutrient in spray

	Mean per plant				Nitrogen			Phosphorus			Potassium			Standard error of increase		
	14 June	12 July	9 Aug.	20 Sept.	12 July	9 Aug.	20 Sept.	12 July	9 Aug.	20 Sept.	12 July	9 Aug.	20 Sept.	12 July	9 Aug.	20 Sept.
Nutrient content* (mg.)																
N	125	479	535	525	91	146	185	17	−23	−14	−9	−11	20	27	35	23
P	12	80	103	100	7	9	7	−1	2	10	3	−8	11	3	6	4
K	153	685	922	963	39	58	77	−45	−27	−17	91	90	249	40	34	33
Na	65	151	186	187	16	13	1	0	−4	−1	−5	−21	0	8	9	9
Ca	58	407	602	607	74	78	86	7	−4	20	17	−41	4	17	34	35
Dry weight† (g).	2·5	25·6	47·7	57·6	2·5	6·1	5·7	0·1	−1·4	1·5	0·9	−0·5	6·6	1·0	1·9	2·7
Leaf area† (sq. cm.)	358	1,305	1,164	625	240	338	156	29	−1	21	−27	−10	109	60	54	54
NAR† (g. per sq. m. per week for interval ending on date shown	—	83	43	24	3	−1	1	1	−1	−1	2	−2	8	3	5	9

* $t = 2{\cdot}26$ † $t = 2{\cdot}05$

solutions. There was some indication that nitrogen in sprays caused an increase in sodium content at the first two harvests, and potassium in spray significantly reduced the sodium content at the second harvest but had no effect at the last. Calcium content was increased by nitrogen sprays at all harvests, but potassium apparently had a negative, not significant, effect at the second harvest which had disappeared by the last.

In general, treatments which increased dry weight, such as spraying with nitrogen-containing solutions, also increased uptake of other nutrients from the soil. All the effects of nitrogen on nutrient content shown in Table I are positive, though they are not all significant. Increases in sodium and calcium content did not accompany the increased dry weight at the last harvest caused by potassium in sprays, and at the previous harvest there was a decrease in the sodium and, to a less extent, in calcium contents of the plants with high potassium contents. This phenomenon of a high concentration of one base depressing the absorption of other bases is well known (Olsen, 1942). At the final harvest the depressing effect of high potassium concentration in the plant on sodium and calcium absorption was balanced by a tendency for enhanced uptake of these elements arising from increased growth, so that sodium and calcium uptake from the soil was unaffected by potassium-containing sprays.

Dry weight yields from spray treatments. Dry matter per plant, at the end of the experiment, was increased by 110 g. per gramme of nitrogen absorbed from the spray and by 26 g. for the absorption of 1 g. of potassium from the spray. The increase in dry weight obtained by spraying with phosphorus was far below the value required for significance, so that the figure of 150 g. dry matter increase per gramme of phosphorus absorbed from the spray has little meaning. The increases with spraying in nitrogen and potassium contents and, to a less extent, in phosphorus content at the end of the experiment were relatively greater than the corresponding increase in dry weight, so that spraying caused an increase in nutrient content expressed as per cent. of dry matter. The mean nitrogen per cent. of dry matter of plants sprayed with nitrogen was 1·03 and for plants not receiving nitrogen 0·79; for phosphorus the corresponding figures were 0·187 and 0·167, and for potassium 2·00 and 1·54.

Distribution of total nutrient content and dry weight (Table II). The percentage of the total dry weight, nitrogen, phosphorus, and potassium content occurring in the tops at the final harvest was not significantly affected by spraying, but there was some indication that the proportion of an element occurring in the tops was slightly increased by applying the element in spray.

Sugar content (Table II). Nitrogen-containing sprays significantly reduced the sucrose per cent. of fresh weight of the roots at the final harvest, but did not affect the yield of sugar. Phosphorus and potassium in sprays had no significant effect on the final sugar content of the roots.

TABLE II

The effect at the end of the growing season of applying nitrogen, phosphorus, or potassium in sprays on sucrose content of root and distribution of nutrient content and dry weight between tops and roots of sugar-beet

	Mean per plant	Mean increase produced by nutrient in spray			Standard error of increase
		N	P	K	
Sucrose per root* (g.) . .	27·8	−0·8	1·5	2·3	1·5
Sucrose per cent. of fresh weight*	16·3	−1·2	0·5	0·6	0·4
Percentage of total nutrient in tops†					
N	49·2	4·3	−3·2	1·2	2·4
P	50·4	6·2	4·2	−2·4	3·3
K . . . . ,	69·8	−1·1	0·1	2·7	1·2
Percentage of total dry weight in tops*	56·2	2·4	−0·1	0·8	1·5

* $t = 2{\cdot}05$ † $t = 2{\cdot}26$

Net Assimilation Rate (Table I). Net assimilation rate was unaffected by any of the spray treatments, so that the increases in dry weight produced by the sprays can be attributed wholly to their effect in increasing leaf area.

Expt. 2. *Sugar-beet*

Leaf area. The effects of the treatments on the growth of the plants are illustrated by the fortnightly leaf-area measurements (Fig. 1). The differences in leaf area between treatments probably account almost completely for the differences in dry matter yield at harvest, because in Expt. 1 net assimilation rate was not changed by nutrient sprays and in another experiment on similar plants, in which the effects of a complete NPK fertilizer and spray were compared, NAR was unaffected by the spray and only slightly increased by the fertilizer during a short period in the middle of the growing season.

Nitrogen fertilizer greatly increased leaf area from mid-June, 9 weeks after the fertilizer was applied at sowing and 2 weeks after spraying started. Phosphorus and potassium fertilizers had much smaller effects on leaf area; phosphorus caused an increase early in the experiment which disappeared by mid-July, and potassium increased leaf area only when nitrogen and phosphorus fertilizer were also present.

Nitrogen spray more than doubled the leaf area by the end of the experiment, except in the presence of N and absence of K fertilizer when it had no effect. The increases were first noticeable 4 weeks after spraying started. Leaf area was unaffected by phosphorus in sprays. After 4 weeks' spraying potassium spray had increased the leaf area and cured the potash deficiency symptoms (marginal leaf scorch) shown by plants receiving NP fertilizer and sprayed with water, nitrogen, or phosphorus. Potassium in spray, which by the end of July had almost doubled the leaf area of plants receiving NP fertilizer, had no effect on that of plants receiving the other fertilizer treatments.

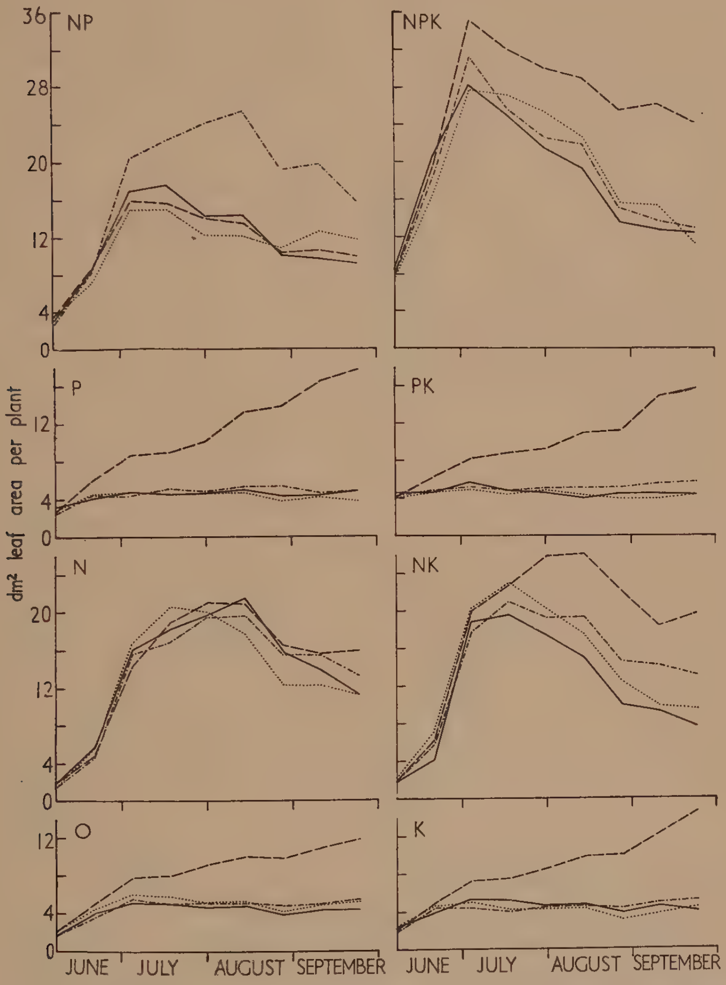

FIG. 1. The effect of nutrient sprays on the leaf area of sugar-beet plants receiving all combinations of presence and absence of nitrogen, phosphorus, and potassium fertilizer added to the soil. Fertilizer treatments are shown by letters.

———— water spray (control) — — — — nitrogen spray
. . . . phosphorus spray –·–·–·– potassium spray

Changes in leaf area, brought about by nutrients applied in leaf sprays and in fertilizers, were mainly the result of changes in leaf size rather than in leaf number, because the effects of treatments on number of surviving leaves were relatively much smaller than their effects on leaf area per plant. Both spray and fertilizer treatments affected leaf number per plant from mid-July onwards; nitrogen had the greatest effect. Table III shows the number of leaves surviving at harvest. Nitrogen fertilizer increased the rate of leaf production more than it increased the death rate, so that the net result was

TABLE III

Mean number of leaves per plant surviving at harvest on sugar-beet plants treated with nitrogen, phosphorus, or potassium fertilizer and sprayed with water or solutions containing nitrogen, phosphorus, or potassium

Mean	35·8
Effect of nitrogen in fertilizer .	6·9
,, ,, phosphorus in fertilizer .	1·0
,, ,, potassium in fertilizer .	3·3
S.E.	±1·3
Sprayed with water . . .	33·4
Sprayed with nitrogen . . .	43·0
Sprayed with phosphorus . .	33·3
Sprayed with potassium. . .	33·4
S.E.	±1·3

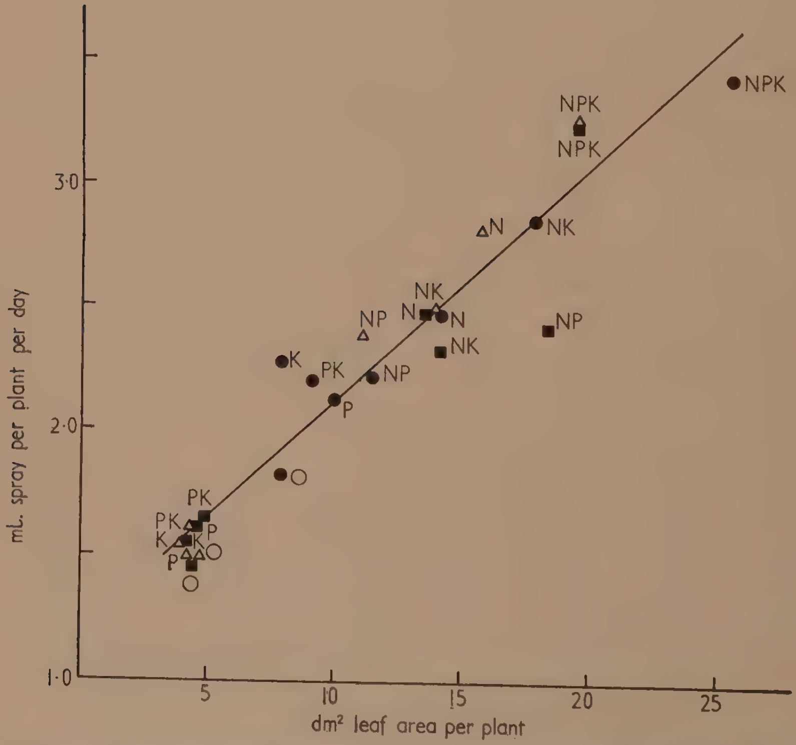

FIG. 2. Relation between mean leaf area per plant for whole experimental period and estimated value of mean volume of spray retained per plant per daily spraying. Fertilizer treatments are shown by letters and spray treatments by symbols. ● — Nitrogen spray, Δ — Phosphorus spray, ■ — Potassium spray. Regression coefficient of volume of spray retained on mean leaf area:— 0·092±0·006 ml.

an increase in the number of surviving leaves. Nitrogen spray, on the other hand, increased only the rate of production.

In order to show the relation between the volume of spray deposited on a plant and its leaf area, the average volume of spray retained per daily spraying and the mean leaf area per plant for the whole growing period were calculated for each treatment from the fortnightly estimations of volume of spray retained by the leaves and leaf area. The highly significant regression coefficient of volume of spray retained on mean leaf area, $0{\cdot}092 \pm 0{\cdot}006$ ml. per day per sq. dm., showed that the volume of spray deposited on a plant was closely dependent on its leaf area. The points plotted in Fig. 2 show that there were no systematic deviations from the regression line associated with the presence of particular nutrients in spray or fertilizer. The linear regression line did not pass through the origin, and the true relation between volume of spray deposited and leaf area per plant is probably a curve rising steeply from the origin, and subsequently less steeply, implying that small plants retained a greater volume of spray per unit leaf area than did large plants. In consequence of this relation between leaf area and the volume of spray deposit, the quantities of nutrient supplied in spray varied greatly with fertilizer treatment.

Nitrogen content. In the first section of Table IV the first column shows the mean nitrogen content for all fertilizer treatments of plants sprayed with water, or with nitrogen, phosphorus, or potassium solutions. The remaining columns show the effects of nitrogen, phosphorus, and potassium fertilizers on the nitrogen contents of plants receiving each of the four spray treatments. The mean nitrogen contents of all the plants and the mean effects of fertilizer nutrients for all spray treatments are given in the last line.

The mean nitrogen content for all spray treatments was increased by nitrogen fertilizer and to a smaller extent by phosphorus and potassium fertilizers. The effect of each of the three nutrients was greater in the presence of one or both of the others than in their absence, that is, all the interactions between fertilizer nutrients (not shown in Table IV) were positive and significant. Nitrogen, but not phosphorus or potassium, in the sprays caused an increase in nitrogen content when the mean values for all fertilizer treatments are considered. It is clear (column 2, Table IV) that the effect of nitrogen fertilizer, but not phosphorus or potassium fertilizer, on nitrogen content was significantly greater for plants sprayed with nitrogen than for plants sprayed with water. The same data are described equally well by the statement that the effect of nitrogen spray was increased by nitrogen fertilizer but unaffected by phosphorus or potassium fertilizers. In general, the differences between the effects of nitrogen, phosphorus, or potassium fertilizer when applied to plants sprayed with water and when applied to plants sprayed with nitrogen, phosphorus, or potassium (columns 2, 3, and 4 of Table IV) are measures of the interactions between fertilizer and spray nutrients. Fertilizer treatment had no effect on the percentage recovery of nitrogen from spray; the mean recovery for all fertilizer treatments was 60 per cent.

TABLE IV

The effect of adding nitrogen, phosphorus, or potassium fertilizer to the soil on the nutrient content of sugar-beet plants, sprayed with water or solutions containing nitrogen, phosphorus, or potassium

Spray treatment	Mean nutrient content (mg. per plant)	Mean increase in nutrient content produced by nutrient in fertilizer		
		Nitrogen	Phosphorus	Potassium
		Nitrogen, mg. per plant		
Water	652	820	163	41
Nitrogen	1,508	1,116	324	107
Phosphorus	699	921	100	111
Potassium	641	828	223	66
S.E.	±34		±67	
Mean	875	921	203	81
		Phosphorus, mg. per plant		
Water	106	110	98	13
Nitrogen	139	105	124	17
Phosphorus	252	236	105	33
Potassium	129	146	126	−5
S.E.	±6		±11	
Mean	157	149	131	15
		Potassium, mg. per plant		
Water	1,100	1,075	156	1,253
Nitrogen	1,379	802	−59	1,541
Phosphorus	1,154	1,204	48	994
Potassium	1,890	1,854	245	1,017
S.E.	±44		±88	
Mean	1,381	1,234	98	1,201

The nitrogen uptakes from spray and fertilizer are compared in Table V. The effects in this table are calculated for each nutrient using the mean values from plants in which the other nutrients are present in all combinations. Thus, the nitrogen content of plants receiving no nitrogen, either as spray or fertilizer, is given by the mean content of the unsprayed plants receiving the fertilizer treatments O, P, K, PK; the content of plants sprayed with nitrogen is the mean for the same fertilizer treatments receiving nitrogen spray; and the content for plants receiving nitrogen as fertilizer is the mean of unsprayed plants of the fertilizer treatments N, NP, NK, NPK. Nitrogen uptake from spray and fertilizer did not differ significantly, nor did the apparent percentage recoveries of 57 and 51 per cent. derived from them. It is possible from a knowledge of the standard error of the uptake to say that these recovery figures do not differ significantly, although as explained on p. 25 the standard error of the recoveries from spray cannot be calculated. The data given in Table V show that nitrogen uptake from fertilizer must exceed $820+3\times 67$ ($=$ 1,021 mg.) in order to differ significantly from the observed value of 820 mg. Since the amount of nitrogen applied in the fertilizer is known exactly, a recovery of less than 64 per cent. based on an uptake of 1,021 mg. is not significantly different from the observed value of 51 per cent. The standard error of the recovery from spray, which depends upon the standard errors of the uptake and estimate of supply, is probably greater than the standard error of the recovery from fertilizer, and so the 13 per cent. taken here as the least significant difference between the recoveries from fertilizer and spray is probably smaller than the true value.

TABLE V

A comparison between the effects of nitrogen, phosphorus, or potassium applied as a leaf spray and as fertilizer added to the soil

	Treatment								
	Nitrogen			Phosphorus			Potassium		
	Spray		Fertilizer	Spray		Fertilizer	Spray		Fertilizer
Increase in nutrient content (mg. per plant) .	708		820	143		98	908		1,253
S.E. . . .		±67			±11			±88	
Percentage recovery in plant . . .	57		51	27		11	54		48
Increase in dry weight (g. per plant) . . .	36·1		74·9	6·6		16·5	7·9		16·2
S.E. . . .		±4·4			±4·4			±4·4	
Increase in dry weight, (g per g. of nutrient absorbed) . . .	51		91	46		169	9		13
Increase in sucrose content of root (g. per plant) . . .	31·1		23·9	3·8		4·8	6·3		11·7
S.E. . . .		±2·9			±2·9			±2·9	
Increase in sucrose per-cent. of fresh weight in root	−1·06		0·46	0·07		0·20	0·98		1·19
S.E. . . .		±0·47			±0·47			±0·47	

Phosphorus content. All three nutrients applied as fertilizer increased the phosphorus content; nitrogen had the greatest effect and potassium least (Table IV). All the interactions between phosphorus and the other nutrients were positive and significant. Phosphorus content was increased by spraying with phosphorus and, to a smaller extent, by spraying with nitrogen and potassium. There was no interaction between phosphorus in spray and in fertilizer, but nitrogen fertilizer increased absorption of phosphorus from the spray. The percentage recovery of phosphorus from the spray was increased by nitrogen fertilizer from 21 per cent. to 30 per cent. This increase was statistically significant when compared with an estimate of error derived from the variances for first- and second-order interactions in the factorial analysis.

Phosphorus uptake from spray was significantly greater than from fertilizer and the percentage recovery from spray was over twice that obtained from fertilizer (Table V).

Potassium content. Potassium content was increased by nitrogen and potassium fertilizer, and to a smaller extent by phosphorus fertilizer (Table IV). All the interactions between potassium and the other nutrients were positive and significant. Potassium in spray increased the potassium content considerably more than did nitrogen; phosphorus had no effect. The increase in potassium content caused by potassium spray was increased by nitrogen fertilizer and decreased by potassium fertilizer. The percentage recovery of potassium supplied in the spray was reduced from 53 per cent. to 30 per cent. by potassium fertilizer and increased from 31 per cent. to 52 per cent. by nitrogen fertilizer. These differences were significant if tested in the same way as were the percentage recoveries of phosphorus from sprays.

There was a smaller uptake of potassium from spray than from fertilizer, but the apparent percentage recoveries were not significantly different (Table V).

Dry weight. Total dry weight per plant at harvest was increased by all three nutrients applied as fertilizer; nitrogen had the greatest effect and phosphorus the least (Table VI). All the interactions between fertilizer nutrients were positive and significant. Nitrogen in sprays, but not phosphorus or potassium, caused an increase in dry weight. The increase in dry weight obtained by adding each nutrient to the soil was slightly less in plants sprayed with that nutrient than in plants sprayed with water. There were no interactions between different nutrients in spray and fertilizer.

Table VI

The effect of adding nitrogen, phosphorus, or potassium fertilizer to the soil on dry weight and sucrose content of the root of sugar-beet plants sprayed with water or solutions containing nitrogen, phosphorus, or potassium

Spray treatment	Mean per plant	Mean increase produced by nutrient in fertilizer		
		Nitrogen	Phosphorus	Potassium
			Dry weight, g. per plant	
Water	65·8	74·9	16·5	16·2
Nitrogen	95·9	62·9	11·4	26·1
Phosphorus	66·1	80·8	4·0	22·1
Potassium	68·9	82·4	14·2	6·6
S.E.	±2·2		±4·4	
Mean	74·2	75·3	11·5	17·8
			Sucrose content, g. per root	
Water	27·5	31·1	4·3	11·7
Nitrogen	38·2	23·1	−0·2	14·9
Phosphorus	28·9	38·1	−0·5	13·3
Potassium	28·9	34·7	4·9	2·0
S.E.	±1·5		±3·0	
Mean	30·9	31·8	2·1	10·5
			Sucrose % of fresh weight	
Water	16·4	0·5	0·2	1·1
Nitrogen	15·1	0·1	−0·7	1·6
Phosphorus	16·1	1·2	−0·7	1·9
Potassium	16·8	0·8	0·6	0·1
S.E.	±0·3		±0·5	
Mean	16·1	0·7	−0·2	1·2

All three nutrients gave greater absolute increases in dry weight when applied as fertilizer than when applied as spray. The increases in dry weight per g. of nitrogen, phosphorus, or potassium absorbed were also greater for fertilizer than for spray nutrients (Table V). There was no further increase in dry weight caused by spraying with a nutrient already supplied as fertilizer, except with nitrogen. The increases in dry weight were always relatively smaller than the accompanying increases in nutrient content, so that all three nutrients, applied either as spray or fertilizer, caused increases in nutrient content per cent. of dry matter in both tops and roots.

Contribution of tops and roots to nutrient content and dry weight per plant

Both tops and roots contributed to the increases in nutrient content and dry weight previously described. The effects of supplying nutrients in sprays or in fertilizers on the percentage of the total nutrient content and dry weight occurring in the tops (fresh tops at harvest plus dead leaves) are shown in Table VII.

The percentage of the total nutrient content occurring in the tops was increased by applying the nutrient in spray for nitrogen, phosphorus, and potassium, and by applying it in fertilizer for phosphorus and potassium. The effect of phosphorus was greater when applied in spray than in fertilizer. None of the treatments altered the distribution of dry matter between tops and roots.

TABLE VII

Percentage of the total nutrient content or of dry weight occurring in the tops of sugar-beet plants receiving nitrogen, phosphorus, or potassium applied as a leaf spray or as fertilizer applied to the soil

Nutrient supplied	Nitrogen		Phosphorus		Potassium	
	N content	Dry weight	P content	Dry weight	K content	Dry weight
In spray . .	61·2	33·0	75·3	32·3	79·4	33·5
In fertilizer . .	56·0	33·8	60·2	33·1	80·1	33·3
Not applied . .	55·1	32·9	45·2	33·6	60·8	33·4
S.E. . .	±1·6	±1·4	±2·3	±1·4	±2·2	±1·4

Sugar content of roots. The yield of sugar per root was increased by nitrogen and to a smaller extent by potassium fertilizer; it was also increased by nitrogen in spray but not by phosphorus or potassium sprays (Table VI). There was some indication that the increases in sugar content caused by applying nitrogen or potassium in the spray were smaller when nitrogen or potassium were also applied as fertilizer, so that potassium spray in the absence of potassium fertilizer gave a small but significant increase in sugar content (Table V).

These increases in yield of sugar were a result of the effect of treatment on both weight of root and concentration of sugar in the root. Sugar per cent. of fresh weight was increased to a greater extent by potassium than by nitrogen fertilizer (Table VI). Nitrogen and phosphorus applied together caused a decrease which may have been related to the symptoms of severe potash deficiency (p. 28) shown by plants that received this treatment. Nitrogen spray caused a reduction in sugar concentration which was less in plants that received nitrogen, phosphorus, and potassium fertilizers applied together than in those receiving the other fertilizer treatments. Potassium spray caused an increase in sugar concentration only in the absence of potassium fertilizer. Comparison of the effects of the three nutrients applied as spray and fertilizer, as described for nitrogen content (p. 32), shows that both potassium spray and fertilizer increased the sugar concentration in the root, nitrogen spray reduced it, and nitrogen fertilizer and phosphorus had no significant effect (Table V).

Expt. 3. Swedes (Table VIII)

Phosphorus applied as both fertilizer and spray increased the phosphorus content of tops and roots. The total phosphorus content was doubled by spraying and more than trebled by application of phosphorus fertilizer. Dry weight was increased only by fertilizer. The apparent percentage recoveries of phosphorus from spray and fertilizer were both about 25 per cent. The distribution of dry weight between tops and roots was unaffected by treatment, but the percentage of the total phosphorus content occurring in the tops was increased by spray and reduced by fertilizer.

TABLE VIII

Effect of fertilizer and spray application of phosphorus to swedes on dry weight and phosphorus content per plant and distribution of phosphorus and dry weight between tops and roots

Treatment	Phosphorus content		Dry weight	
	mg. per plant	Percentage in tops	g. per plant	Percentage in tops
Control	73	30·2	54	34·1
Fertilizer	297	19·1	118	29·2
Spray	141	44·3	54	33·3
S.E.	±7	±1·8	±7	±2·3

DISCUSSION

So far it has been assumed that the observed increase in content of a nutrient caused by a spray or fertilizer treatment supplying that nutrient can be attributed entirely to uptake from the spray or fertilizer, and it is now necessary to consider what errors, if any, are involved in this assumption.

The water in which the leaves of unsprayed plants of Expts. 2 and 3 had been washed at harvest contained negligible amounts of nutrients, showing that there was no appreciable leaching from the inside of the leaves during washing. Experiments to investigate the washing of leaves of sprayed plants showed that negligible amounts of nutrient were removed by washings after the first, so it is probable that none of the estimated nutrient content was external deposit. (This is not true for the dead leaves, see p. 24.) It is therefore unlikely that the washing of the leaves introduced any serious error in the estimate of nutrient content.

The increase in content caused by spraying with a particular nutrient is only an accurate measure of uptake from the spray if absorption of that nutrient by the roots is unaffected by the spray treatment. The factorial design of Expt. 1 permitted uptake of a nutrient from spray to be measured from the difference in content between plants which had identical treatments except in respect of the nutrient under consideration. Clearly, therefore, this difference accurately measured uptake from the spray even when other nutrients in the spray solution affected absorption by the roots of the nutrient being measured. Such complications were avoided in the second and third experiments by applying the nutrients singly in the spray solutions. So, in all three experiments, increased nutrient content can be taken as a reliable measure of uptake from the spray provided that absorption of a nutrient by the leaves did not affect uptake of the same nutrient by the roots. Experiments not yet reported, in which the supplies of a nutrient to the leaves or roots were distinguished by labelling one or other with an isotope, have shown that under some conditions such an interaction occurs but that in the present experiments it was unlikely to have introduced a serious error in estimating nutrient uptake from sprays.

The factorial arrangement of fertilizer treatments in Expt. 2 allowed uptake of a nutrient from fertilizer to be estimated independently of any effect of other fertilizer nutrients on uptake from the soil of the nutrient in question, from the mean increase in content of that nutrient caused by its application in fertilizer, averaged over all other fertilizer treatments.

Nutrient uptake from spray. The combined results of the three experiments prove that nitrogen, phosphorus, and potassium can be absorbed by the leaves of plants from sprays. An increase in nutrient content of roots or an increase in dry weight, caused by applying a nutrient in a leaf spray, proves that the nutrient in question has been absorbed by the leaves. Increased content of tops alone, with no accompanying increase in dry weight, might be due to a persistent external deposit of salt, not removed by the pre-harvest washing. The first experiment proved that nitrogen and potassium were absorbed by leaves; spraying with solutions containing nitrogen or potassium increased plant dry weight and the content of the applied nutrients in tops and roots. Phosphorus in sprays caused no increase in dry weight and most of the increase in phosphorus content was in the tops, so that phosphorus uptake by the leaves was not definitely established. This was done by Expts. 2 and 3. In the second experiment only nitrogen in sprays increased dry weight but all three nutrients caused increases in content of roots and tops, which shows that all three were absorbed by the leaves. Phosphorus sprays in Expt. 3 did not affect the dry weight of swedes but increased the phosphorus content of the roots from 48 to 78 mg. per plant.

Absorption of one nutrient by the leaves was unaffected by the presence of other nutrients in the spray, but uptake of a nutrient from the soil was sometimes influenced by other nutrients in the spray. Nitrogen in spray increased the uptake of phosphorus and, to a smaller extent, of potassium from the soil, and potassium in the spray slightly increased the uptake of phosphorus from the soil. Therefore increase in nitrogen content caused by complete NPK sprays in previous experiments (Thorne, 1954) was probably almost entirely due to uptake from the spray, but some of the increase in potassium content and most of the increase in phosphorus content were due to absorption from the soil. The increases in dry weight and leaf area observed in these experiments were probably due mainly to the nitrogen in the spray solution.

Interaction between spray and fertilizer nutrients. The increases in nitrogen, phosphorus, and potassium contents caused by spraying with these nutrients in Expt. 2 were all greater in plants that had received nitrogen fertilizer. Figs. 2 and 3 show that this was mainly due to the greater quantities of spray nutrients that these plants retained because of their greater leaf area. The volume of spray retained by the leaves of a plant and the quantities of nutrients absorbed from the spray are both related linearly to leaf area, when this is changed by fertilizer treatment. The different slopes of the regression lines for nitrogen, phosphorus, and potassium uptake against leaf area are largely accounted for by the different concentrations of the three nutrients in the sprays; the phosphorus spray was the least concentrated and the potassium spray was the most concentrated.

The large spread of the points about the regression line for plants sprayed with potassium (Fig. 3) indicates that fertilizer did not affect uptake of potassium from spray only through changing leaf area per plant. Change in leaf area, and hence in quantity of nutrient retained by the leaves, accounts entirely for the effects of fertilizer on uptake from sprays only when the apparent percentage recoveries in the plant of nutrients applied in sprays are unaffected by fertilizer treatments. Nitrogen fertilizer had no effect on the percentage recovery of nitrogen from spray, but increased that of phosphorus and potassium. The increased uptake of nitrogen from spray caused by nitrogen fertilizer was therefore entirely due to the larger quantities of nutrient retained by the larger leaves, but increased efficiency of absorption as well as an increase in the quantity of nutrients retained by the leaves appears to have contributed to the increased uptake of phosphorus and potassium from sprays in plants that received nitrogen fertilizer. The percentage recovery of potassium from spray, but not the estimated amount of potassium retained by the leaves, was reduced in plants that received potassium fertilizer. The decreased uptake of potassium from the spray by these plants therefore appears to be due entirely to less efficient absorption from the spray. The same results would have been obtained if uptake from the spray remained unaltered, but plants sprayed with potassium absorbed less potassium through the roots than those sprayed with water. Without labelling the nutrient either in the spray or in the fertilizer, for example with a radio-active isotope, it is not possible to determine whether nutrient absorption by the root reduced uptake by the leaves or vice versa. However, in view of the discussion below on the meaning of the percentage recovery figures, the latter explanation appears more probable.

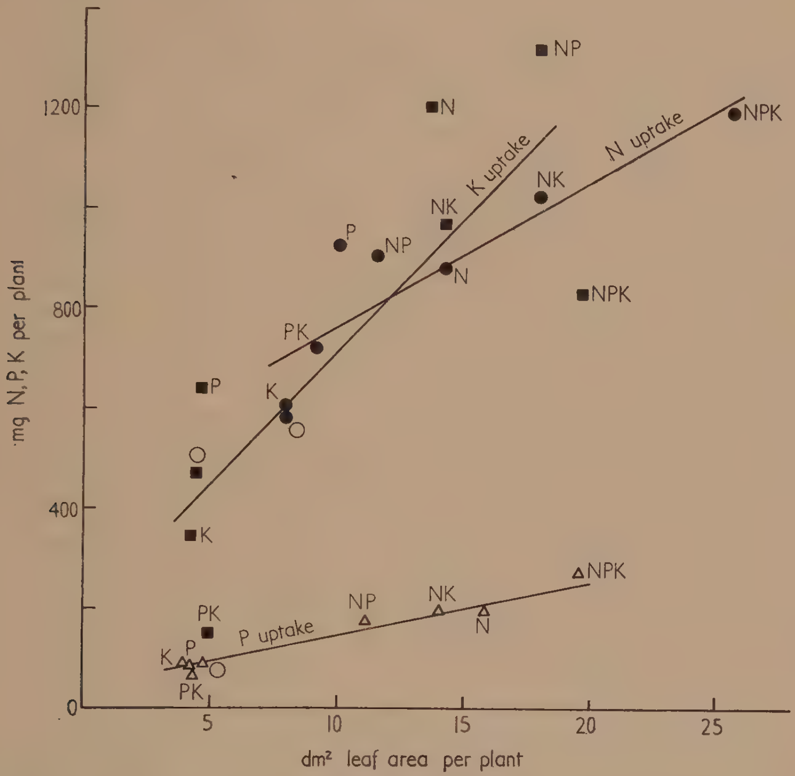

FIG. 3. Relation between mean leaf area per plant for whole experimental period and uptake of nitrogen, phosphorus, or potassium from leaf sprays. Fertilizer treatments are shown by letters and spray treatments by symbols. ● — Nitrogen spray, △ — Phosphorus spray, ■ — Potassium spray. Regression coefficients of nutrient uptake on leaf area are:—

N, 29·3±2·3   P, 11·2±1·4   K, 53·5±13·5

The interactions of spray and fertilizer nutrients in this experiment are compatible with those described previously (Thorne, 1954) where NPK fertilizer increased the uptake of all three nutrients from NPK sprays, probably by acting in an analogous manner to nitrogen fertilizer in the present experiment. The uptake of potassium from NPK spray was not reduced by NPK fertilizer, but the depression of potassium uptake from spray produced by potassium fertilizer in the present experiment, averaging all fertilizer treatments, did not appear when NPK fertilizer was compared with no fertilizer.

Comparison of the effects of spray and fertilizer nutrients. Larger increases in dry weight per plant were always obtained from nutrients applied in fertilizer than in sprays. No doubt this was partly because plants absorbed more nitrogen and potassium from fertilizer than they did from spray, but a comparison of dry weight increase per unit of nutrient absorbed from spray or fertilizer shows (Table V) that nutrients applied in fertilizer were far more effective in increasing dry weight than those applied in spray. This is probably due to the later uptake of the spray nutrients; the fertilizer nutrients were all applied at sowing and those in the spray were supplied continuously throughout the growing season. Field experiments showed that no such difference occurred when the soil and spray applications were made at the same time (Thorne and Watson, 1952).

As equal quantities of nitrogen, phosphorus, and potassium were not supplied in sprays and fertilizer, the percentage recoveries in the plant of nutrients applied by the two methods must be used to compare the efficiencies of uptake from spray and fertilizer. The apparent percentage recoveries, based on the estimated quantities of nutrients retained, were used for this purpose (p. 25). The percentage recoveries (Table V) for nitrogen and potassium from spray and fertilizer were all about 50 per cent. The recovery of phosphorus from spray was 27 per cent. and from fertilizer 11 per cent.

There was a tendency for a greater proportion of the increased content to occur in the tops when nutrients were applied in spray than when applied in fertilizer. In Expt. 1 spraying caused small and insignificant increases in the proportion of the total nutrient content occurring in the tops, but in Expt. 2 this proportion was significantly increased by spraying with all three nutrients. It was unaffected by nitrogen fertilizer, increased slightly by phosphorus fertilizer, and potassium in fertilizer had the same effect as in spray. Fifty-nine per cent. of the increased phosphorus content caused by spraying the swedes of Expt. 3 occurred in the tops, but only 16 per cent. of the increase caused by fertilizer application of phosphorus was in the tops; i.e. there was a tendency for phosphorus to accumulate in the part of the plant it entered. The distribution of dry weight between tops and roots was never affected by spray or fertilizer application of any nutrient.

Validity of estimates of percentage recovery of nutrients. The accuracy of all the recovery figures depends on that of the estimates of nutrient uptake and quantity of nutrients supplied; the former have been discussed already. The quantities of nutrients applied as fertilizer were known exactly, so that the percentage recoveries from fertilizer given above have errors which depend only on those for nutrient uptake and are good estimates of the recovery in the plant of nutrients added to the soil.

The quantities of nutrients retained by the leaves from sprays could not be determined directly but were estimated by the method described on p. 23. It is important to know the random errors involved in this estimate and how closely the estimate approximates to the true value, because all the apparent percentage recoveries from sprays were based upon it. The standard errors of the estimated quantity of nutrients retained by the leaves could not be calculated (see p. 23), but were probably not large because, in another experiment on similar plants in which the estimates of volume of spray retained were made in duplicate on groups of 4 plants, the coefficient of variation for the volume of spray retained per day per plant was only 8 per cent. The similarity of the volume of spray retained by different plants of similar leaf area (Fig. 2) also indicates that the errors in making this estimate were not excessive. If the estimated quantity of nutrient retained by the leaf is assumed to be near the true value, the maximum recovery obtained from spray was only about 60 per cent., and it is necessary to account for the remaining 40 per cent. of the nutrients applied to the leaf. These were not deposited on the leaf surface, because only small amounts of nutrient were found in the solutions used for washing the tops at harvest, and, as there was no run-off, nutrients deposited on the leaf at one spraying could not have been washed off at the next. It therefore appears that most of the nutrient retained by the leaves from sprays must be absorbed and that low apparent percentage recoveries are due either to reduced absorption by the roots of nutrients applied in sprays or to an over-estimation of the volume of spray retained by the leaves.

If it is assumed that apparent recoveries of about 60 per cent. represent true recoveries of 100 per cent., there remains the problem of accounting for recoveries of under 60 per cent. For example, in the present sugar-beet experiment the apparent recoveries of potassium from the sprays were 52 per cent. and 29 per cent. respectively, in the presence and absence of potassium fertilizer, and the apparent recovery of phosphorus from spray was 27 per cent. These different recoveries were obtained from plants that had mean leaf areas for the whole season of 10·1, 10·7, and 9·6 sq. dm. per plant, and that received an average of 2·0, 2·2, and 2·1 ml. of spray per day. It has been shown that the estimated volume of spray retained by the leaves of the plant depends on its leaf area, and it is reasonable to assume that the bias in making this estimate was the same for plants of similar leaf areas. It is unlikely therefore that the two lower recoveries were due to unusually large bias in estimating the quantity of spray applied, but they may have been due to reduction in uptake from the soil of the nutrient in the spray.

The effect of nitrogen fertilizer on the recovery of phosphorus and potassium from sprays may have been due to a difference in bias in estimating the volume of spray retained by the widely different leaf areas of plants with and without nitrogen fertilizer. However, this appears unlikely because in the graph of estimated volume of spray retained by the leaves against leaf area (Fig. 2) the points for plants with and without nitrogen fertilizer lie close to the same line. An alternative explanation of the apparent increased recovery of potassium or phosphorus from the spray in plants that received nitrogen fertilizer is that the potassium or phosphorus uptake from the soil caused by applying nitrogenous fertilizers was greater in plants sprayed with these nutrients than in plants sprayed with water. It is not possible to decide from the present experiment if either of these explanations is correct.

From this discussion of the errors involved in estimating the uptake and supply of the nutrients from sprays, it appears probable that when nutrients are applied singly most of the nitrogen and potassium deposited on the leaves is absorbed. Phosphorus, and potassium applied to plants with high potassium to the soil, may also be completely absorbed, but if so, there must be an accompanying depression of uptake by the roots.

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Metabolism of Radioactive Sugars by Tobacco leaf Disks

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SUMMARY

Destarched tobacco-leaf disks were floated on 5 per cent. (w/v) solutions of sucrose uniformly labelled with ^{14}C in either the glucose or fructose moiety, and on invert sugar in which one hexose only was so labelled. The experiments were carried out in an atmosphere of oxygen at 25° C. Seventy-five per cent. of the sugar lost from the external solutions was recovered as starch, sucrose, fructose, glucose, and CO_2. With sucrose as the substrate, 30 per cent. of the material was recovered as CO_2, and 17 per cent. each as starch, sucrose, fructose, and glucose. With invert sugar as the substrate, 30 per cent. was again recovered as CO_2, only 20 per cent. as the three sugars together, and 50 per cent. as starch. Whichever hexose was initially labelled and whether the sugar was supplied as sucrose or hexose, the relative specific activities of starch and sucrose in the leaf disks and of the CO_2 evolved were equal or nearly equal to that of the sugar supplied. With sucrose as the substrate the sucrose in the disks retained its asymmetry of label, and free hexoses produced were similarly asymmetrically labelled. When invert sugar was the substrate the sucrose synthesized was strongly labelled in both moieties, as also were the free hexoses. It is concluded that fructose and glucose free or combined in sucrose were equally available for starch synthesis and CO_2 formation, and that there can be no question of preferential utilization of one or other hexose. Starch and CO_2 must arise from a common source in which readily formed derivatives of the hexoses are rapidly equilibrated. Free hexose cannot participate directly in either sucrose or starch synthesis. Accumulation of sugar not immediately metabolized and inversion of sucrose take place at a site remote from the common pool. A scheme to accommodate the results is discussed.

INTRODUCTION

THE close relationship between starch synthesis and dissolution, sucrose synthesis, and fructose and glucose interconversion was established by early experiments in which detached leaves or pieces of leaves were floated on sugar solutions in the dark, by studies of diurnal fluctuations of starch and sugars in leaves and of starch synthesis and dissolution in fruits and tubers. The subject has been reviewed by Nurmia (1935), Hassid and Putman (1950), and by Porter (1953). As a result of numerous experiments it is well established that starch formation can be induced, in the presence of oxygen, by

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supplying leaves with sugar solutions in concentrations ranging from 0·1 to 10 per cent., and that when hexoses are the substrate there is always concomitant synthesis of sucrose. There are also records of starch synthesis from sugars other than glucose, fructose, and sucrose, and from related compounds. In spite of the recent great advances in knowledge of enzyme mechanisms concerned in carbohydrate metabolism, the precise transformations undergone by sugars in leaves leading to starch formation are still largely a matter for speculation, more especially as regards the appearance of the fructose structure and the reactions requiring oxygen.

The advent of chromatography and the use of isotopes has led to a revival of the sugar-feeding method as a line of approach to the problems of sugar interrelationships. Investigation by this method involves the use of living tissue and the consequent utilization of sugar substrates in respiration and perhaps also in reactions other than direct carbohydrate interconversions. With the new techniques it is possible to follow in detail the contributions to starch synthesis and to respiratory carbon dioxide of the individual hexoses or of the moieties of sucrose, when these sugars are supplied to leaf tissues.

An attempt has now been made to follow quantitatively the fate of radioactive sucrose or invert sugar supplied to destarched tobacco-leaf disks under controlled environmental conditions. To this end sucrose was prepared which was uniformly labelled with ^{14}C either in the glucose or the fructose moiety. These two types of sucrose, and also invert sugar in which one hexose only was uniformly labelled with ^{14}C, were supplied to leaf disks enclosed in Warburg flasks. Gas exchange was measured over 24-hour periods and at suitable time intervals flasks were removed for analysis of the contents and for determination of the redistribution of labelling between sugar, starch, and carbon dioxide.

Results of some preliminary experiments of this type have already been mentioned (Porter, 1953) and a short account of the experiments here recorded was given to the Second Isotope Conference, Oxford (1954).

MATERIALS AND METHODS

Preparation of sugars labelled with ^{14}C

Glucose uniformly labelled (^{14}C–G) was the preparation described by Porter and Martin (1952). Activity about 5·5 μc. per mg.

Fructose uniformly labelled (^{14}C–F) was purchased from the Radiochemical Centre, Amersham, Bucks. Activity 50 μc. per mg.

Sucrose labelled in the fructose moiety (S/^{14}C–F) was prepared from inactive sucrose and uniformly labelled fructose by enzymic transfer of glucose using sucrose phosphorylase (Wolochow, Putman, Doudoroff, Hassid, and Barker, 1949).

A culture of *P. saccharophila*, obtained from Dr. W. Z. Hassid, was maintained by monthly subculturing, on a sucrose mineral medium (Hassid and Doudoroff, 1950). For the preparation of sucrose phosphorylase 20 litres of medium, in which sucrose concentration was 0·3 per cent. (w/v), were inoculated with a 20-ml. culture of the bacterium. The large-scale culture was grown in an iron tank (Arnstein, Cook, and Lacey, 1948) held at 20° C. for 3 days. The cells were harvested by centrifugation, washed three times with water, and the wet cells (75 g.) crushed in a bacterial press (Hughes, 1951) at −20° C. This procedure ruptured over 90 per cent. of the cells and yielded an enzyme preparation with double the activity of one prepared by grinding with sand. The crude extract was purified by the procedure of Hassid and Doudoroff (loc. cit.). Precipitates were collected by centrifugation at 20,000 g. and all operations conducted at 3° C. The final solution in citrate buffer was freeze dried and used for the preparation of S/^{14}C–F. Tests of activity of sucrose phosphorylase were made as purification proceeded by measuring formation of glucose-1-phosphate from sucrose in the presence of phosphate. Full details of this preparation and of the tests of activity have been recorded by May (1954).

For the synthesis of S/^{14}C–F a digest containing 250 mg. of sucrose, 1 mg. of uniformly labelled fructose containing about 50 μc., and 95 mg. of the sucrose phosphorylase preparation in 3·2 ml. of citrate at pH 6·64, was incubated at 28° C. for 24 hours, when the reaction was stopped by heating to 52° C. The course of reaction was followed by withdrawing samples of 4 μl., separating the sugars chromatographically, and estimating radioactivity by counting the spots on the paper (Edelman, 1954). After 24 hours 90 per cent. of the radioactivity had become incorporated in sucrose. The sugars in the digest were then separated by passage through a charcoal/celite column (25 mm. × 160 mm.) containing 35 g. of the mixture (Whistler and Durso, 1950). Monosaccharides were eluted with water and subsequently sucrose with 10 per cent. alcohol (v/v), elution being followed by radioactivity measurements. The fractions containing sucrose were combined and concentrated, and yielded 249 mg. of syrup containing about 45 μc. The mean of four estimates of the activities of the fructose and glucose components showed that only 1·5 per cent. or the total activity was in the glucose component.

Sucrose labelled in the glucose moiety. (S/^{14}C–G) was prepared from inactive sucrose and uniformly labelled glucose by enzymic transfer of fructose using sucrase (Bealing, 1953; Edelman, 1954). A preparation of sucrase from *Aspergillus oryzae* was obtained from Dr. F. J. Bealing. The synthesis of S/^{14}C–G was carried out using three successive digests. To 0·5 ml. of the sucrase preparation was added 0·5 ml. of 0·05 M. acetate at pH 5·0, 90 mg. of sucrose, and 50 mg. of ^{14}C–G containing about 250 μc. After incubation at room temperature for 2 hours the reaction was stopped by immersing the tube containing the digest in boiling water for 5 minutes. Samples of 5 μl. were withdrawn for chromatographic examination and radioactivity measurements as before. 15 μc. of the activity had been transferred to sucrose. The sugars were separated on a charcoal column as already described, except that sucrose was eluted with 5 per cent. alcohol (v/v) to prevent contamination with oligosaccharides produced simultaneously by the sucrase action. These materials were subsequently removed from the column with 10 per cent.

alcohol (v/v). The hexose fractions were concentrated yielding 84·6 mg. of syrup, which contained products of inversion of sucrose in addition to the residual ^{14}C–G. To this hexose was added sucrose, acetate buffer, and sucrase as before and the incubation and separation procedures repeated, resulting in a further transfer to sucrose of 20 μc. The whole process was repeated a third time starting with 116 mg. of hexose, of which 43 mg. was residual ^{14}C–G. 12·5 μc. was transferred to sucrose. The three fractions containing S/^{14}C–G were combined and concentrated yielding 143·5 mg. of syrup containing about 45 μc. The varying yield in the successive digests is probably attributable to the fact that incubation was carried out at room temperature. The mean of four estimates of the activities of the sucrose components showed that only 2·6 per cent. of the total was in the fructose component.

Preparation of leaf samples for analysis

Leaf disks on removal from sugar solutions were washed with water, dried on blotting-paper, and at once transferred to boiling 90 per cent. alcohol (v/v). After extraction in a Soxhlet apparatus starch was isolated from the alcohol insoluble material and the sugars separated from the alcoholic extract.

Isolation of starch

Starch was isolated by the method of Hanes (1936), adapted to a micro scale. Alcohol insoluble material from about 180 mg. F.W. was ground for 10 minutes with a few grains of sand and 0·25 ml. of water. The fine cell suspension resulting was transferred to a small boiling tube with a ground-glass socket, using 4·75 ml. of absolute alcohol. One drop of concentrated HCl was added and the mixture refluxed for 12 minutes. The acid alcohol was removed using a micro filter stick and the residual material washed three times with 95 per cent. alcohol (v/v). Starch was extracted from the cellular material by boiling with successive additions of 4 ml. of water for 5 minutes, until the cell debris gave no colour with iodine; usually a total of 16 ml. of water sufficed. The cell debris was finally separated by centrifugation. The aqueous extract was made alkaline with 1 drop of 2N NaOH, concentrated to about 1 ml. on a water-bath and then transferred to a centrifuge tube in a final volume of 3 ml. Absolute alcohol (7 ml.) was added and after 24 hours the precipitated starch was separated by centrifugation, washed with alcohol, and dried *in vacuo*. The product was usually completely soluble in hot water; any traces of insoluble material were rejected.

Estimation of starch by β-amylolysis. β-amylase was prepared by the method of Hopkins, Murray, and Lockwood (1946), and the final solution freeze-dried. Starch solution containing about 250×10^{-2} mg. was added to 0·1 ml. of 0·6 per cent. β-amylase in 0·1 M. acetate at pH 4·6. The volume of the digest was usually 10 ml., but if there was only a small amount of starch the volume was reduced to the lowest practical limit. The digests were incubated for 24 hours at 30° C., under toluene. A second addition of enzyme was then made and incubation continued for a further 24 hours. The maltose produced was estimated by copper reduction (Nelson, 1944; Somogyi, 1952). Percentage conversion to maltose was 51 to 53. The amount of starch was calculated by reference to a similarly treated solution prepared from starch isolated in granular form from tobacco leaves (Porter and Martin, 1952), and carefully purified and dried.

Estimation of starch by the anthrone method. The procedure of Seifter, Dayton, Novic, and Muntwyler (1950) was used, with the modification of the heating conditions to 2 hours at 78° C. instead of 10 minutes at 100° C.

In Table I*a* estimates of starch in the hot water extract of the leaf material are compared with the weights of starch isolated. The purity of the isolated material was further tested by measuring the relative specific activity before and after reisolation from the iodine complex (Putman, Hassid, Krotkov, and Barker, 1948). As shown in Table I*b* no change was observed. Finally estimation of starch by β-amylolysis and by the less specific anthrone method showed that little contaminating carbohydrate could have been present, estimates by anthrone being in general about 10 per cent. higher than those by β-amylase.

TABLE I

a. Estimates of starch in tobacco-leaf disks by β-amylolysis, and amounts isolated (mg.) (see text)

b. Relative specific activities c.p.m./10^{-3} mg. of starch isolated from tobacco-leaf disks before and after purification by means of the iodine complex

Sample	*a.* Estimated	Isolated	*b.* Untreated	Iodine treated
1	11·0	10·8	4·7	4·1
2	10·5	11·0	5·9	5·7
3	3·3	3·4	2·2	2·2
4	2·8	3·2	2·2	2·6

Isolation and estimation of sugars and preparation of samples for counting

The procedure to be described was designed to permit estimates of amount and counts of activity to be made on the same sample of each sugar after chromatographic separation. Replicated estimates of relative specific activities obtained in this way lay within ±10 per cent. of the mean values.

The alcohol extracts of the leaf disks, about 30 ml., were evaporated to about 5 ml. and then dried *in vacuo*. The dry residue was dispersed in toluene and 1 ml. water added. The mixture was centrifuged and the clear aqueous layer containing sugars transferred quantitatively to paper for chromatographic separation. Solutions were transferred by means of an Agla micrometer syringe, up to 100×10^{-3} ml. being applied to give a spot of diameter 1 cm. 5×10^{-3} ml. were transferred at a time, and dried in a stream of hot air before the next application. 0·5 ml. of each solution was applied thus in 5 spots, 1·25 cm. apart. The papers were developed with butanol/acetic acid/water (Partridge, 1948) for 60 to 72 hours. The positions of the separated sugars were located by spraying guide strips with benzidine (Bacon and Edelman, 1951), and the corresponding strips of the sections cut out. The strips were eluted with water and the eluate delivered on to stainless steel counting plates using the apparatus illustrated in Fig. 1. Ninety per cent. of added sugar was recovered in the water eluates. After counting (see below) the plates were placed in a known volume of water and allowed to stand for 2 hours to dissolve the sugar. Aliquots were then withdrawn for estimation of glucose (Nelson, 1944; Somogyi, 1952) and of fructose and sucrose (Bacon and Bell, 1948). For examination of its component hexoses, sucrose was inverted by addition of 0·04 ml. of sucrase (B.D.H. invertase concentrate) to 1 ml. of sucrose solution containing 50 to 300×10^{-3} mg., and the mixture left to stand at room temperature for 2 hours. Aliquots of the inverted solution were transferred to paper and after chromatographic separation of the fructose and glucose these sugars were eluted, estimated, and their radioactivity measured. When using the copper reduction method rather high blank values were obtained, due to reducing material in the paper. It was not therefore advisable to use this method for amounts of sugar less than about 10 μg.

Estimation of carbon dioxide

Carbon dioxide was estimated by the direct method of Warburg (see Umbreit, Burris, and Stauffer, 1945). This method assumes that output is not affected by the presence or absence of CO_2 in the tissue environment. In the present experiments CO_2 concentrations rose to 12 per cent. when NaOH was absent from the centre wells of the Warburg flasks. Respiratory quotients were always a little in excess of 1·0, suggesting a possible over-estimation of CO_2. Runeckles (unpublished) has therefore estimated carbon dioxide trapped in the centre well, and from his results it appears that the direct method is not here in error by more than 3 per cent. There is not therefore evidence for an effect of accumulating CO_2 upon the respiration rate.

Radioactivity measurements

All samples were counted at infinite thinness, the material being spread on stainless steel plates and placed 2 mm. from the window of a thin end-window G/M counter. The plates were rimless and sharp edged and held 0·2 ml. of liquid by surface tension, so adherence of solute to a rim was avoided. For application of samples to the plates, these were first covered with 0·2 ml. of 0·1 per cent. stergene and dried under an infra-red lamp in a stream of air to minimize concentration at the plate edge. The radioactive solution was then applied by dripping from paper (see above and Fig. 1) or by means of the Agla syringe. After drying, the solutes were redispersed with 0·1 per cent. stergene and again dried to ensure even spreading. Counts in 20 minutes were recorded if activity was low, or, with higher activity, the time for 2,000 counts. Using these metal plates, five times as much material can be counted as can satisfactorily be run to give a single spot for counting on paper. With the elimination of absorption by the paper as well the plating technique increases sensitivity about ten times as compared with paper counting.

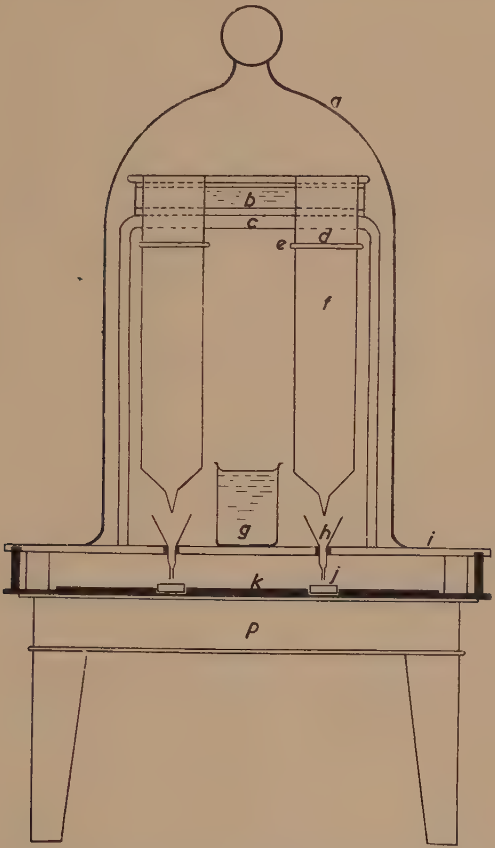

FIG. 1. Apparatus for transferring sugars separated by paper chromatography on to metal counting plates. Paper strips (*f*) cut from chromatograms are attached to paper wicks (*d*) by glass rods (*e*) held by rubber bands. The wicks dip into a water reservoir (*b*) which is supported on a glass framework (*c*). The atmosphere is kept saturated by covering with the bell jar (*a*), and evaporation of warm water from the beaker (*g*). Eluate drops from the paper strips into funnels (*h*) inserted in a perspex base (*i*) and on to metal plates (*j*) resting in depressions in a copper plate (*k*). The plate is warmed to about 60° by a hot plate (*p*) so that each drop of eluate evaporates from the counting plate before another falls. The apparatus carries 8 strips at a time.

The procedure was slightly modified for preparing samples for carbonate counting, because of the rapid exchange between atmospheric CO_2 and carbonate. Excess alkali was used to absorb CO_2 to ensure that only carbonate was present, since it is much more stable in respect of exchange than the bicarbonate. 100×10^{-3} ml. of carbonate solution was transferred to stergene-treated counting plates and at once dried *in vacuo*. Counting was carried out within 2 hours, but no loss of activity was in fact detected after 48 hours *in vacuo*. The counting rate in air did not alter measurably during the maximum counting time of 20 minutes.

Method of sugar feeding

Tobacco plants with 4 or 5 fully expanded leaves were kept in darkness for 60 hours. In this time the second and third leaves below the apex became completely destarched, and total sugar concentration was reduced to a very low level (less than 2 per cent. of the dry weight). Leaf disks, 1 cm. diameter, were punched from such leaves, dried gently between filter papers and divided randomly into sets of 12, which were placed in Warburg flasks. Each flask contained 1 ml. of 5 per cent. (w/v) sugar solution or water and the disks were arranged in two layers. 0·2 ml. 10 per cent. (w/v) NaOH was introduced into the centre well, except for one vessel of each treatment to enable direct estimates of respiration to be made manometrically. The flasks were gassed with oxygen, a procedure found to increase the amount of starch formed, and equilibrated with the bath temperature of 25° C. to 26° C. The shaking rate was 80 to 100/min. Manometric readings were started 1·5 hours after the disks were punched out, and were continued hourly. Flasks were removed after the first reading and then after 8, 16, and 24 hours, and the contents prepared for analysis and counting as already described.

TABLE II

Q_{O_2} values (µl. O_2/mg. D.W./hr.) for tobacco-leaf disks floated on water and 5 per cent. sucrose solutions in air and oxygen

		$-Q_{O_2}$		
Shaking rate	Time (hrs.)	Water and air	5% Sucrose air	oxygen
80/min.	0–4	2·5	3·1	5·8
	18–22	1·9	2·9	8·0
120/min.	0–4	2·5	3·4	6·1
	18–22	1·7	3·3	8·6

When sucrose was supplied to leaf disks in this way there was some production of reducing sugar in the external solution. Preliminary experiments showed that the amount of inversion depended upon the area of cut surface, and was increased by prolonged preliminary washing of the disks. It also depended on the age of leaf, but not upon degree of aeration. High external pH (7 to 8) reduced inversion, but as well adversely affected starch synthesis, so that no advantage could be obtained in this way. No conclusive evidence was obtained as to whether inversion was brought about by sucrase at the cut edges, or by micro-organisms adhering to the leaf surface (Weatherly, 1953). However, it was shown that when 1-cm. diameter disks cut from the third leaf below the apex of a destarched tobacco plant were floated on neutral

5 per cent. sucrose solution there was about 2 per cent. hydrolysis in 24 hours, and that the amount increased with time of exposure to the sucrose solution.

In oxygen the respiration rate was increased above that in air by about 100 per cent., and the endogenous respiration rate in air was about 25 per cent. of that in sucrose and oxygen. An example of the rates of oxygen uptake when disks were floated on sucrose is given in Table II. Increasing the shaking rate had little effect on uptake, indicating that the rate of equilibration of solution and gas phase was not limiting oxygen uptake. Using oxygen as the gas environment had the additional advantage of inducing greater homogeneity between disks in respect of the amount of starch formed.

RESULTS

Four experiments are recorded in which 5 per cent. sucrose labelled in either the fructose or glucose moiety, and 5 per cent. invert sugar in which one hexose was labelled were supplied to tobacco leaf disks. The sugar solutions contained approximately 4 μc. of ^{14}C per ml.

Carbohydrate changes in external solutions and in leaf disks

As an example of the type of data collected detailed results for one experiment, that in which ^{14}C–F+G was supplied are presented in Table III. Attention is drawn to the fact that when NaOH was absent from the centre well, although total uptake was unaffected, there was less starch formed and more sugar accumulated than when CO_2 was continuously removed. This effect was a constant feature of this type of experiment, but at present no explanation for it can be offered. It has already been pointed out (p. 48) that CO_2 output is not affected.

TABLE III

a. Estimates of sugar and starch (N *by anthrone*, B *by β-amylolysis*) *in tobacco-leaf disks floated for 24 hrs. on 5 per cent. invert sugar in Warburg flasks gassed with oxygen, and of the carbon dioxide evolved*

b. Estimates of sugar in the external solution. Sugar supplied ^{14}C–F+G

P. NaOH present in centre well. *A.* NaOH absent from centre well. Temp. 25° C.

	Time (hrs.)	*a.* Fructose	Glucose	Sucrose	Starch		CO₂ (as hexose)	Total	R.Q.	*b.* Fructose	Glucose	Sucrose	Total
		mg. per 12 disks			*N*	*B*					mg.		
P	0	0·15	0·06	0·24	—	—	—	0·45	—	24·4	24·1	0·0	48·5
	8	0·77	0·39	0·56	0·98	1·04	0·84	3·54	1·10	22·6	21·1	0·1	43·8
	16	0·92	0·51	0·69	2·36	2·47	1·99	6·47	1·11	20·6	16·2	0·2	37·0
	24	1·00	0·68	0·77	4·12	3·35	3·61	10·18	1·09	18·4	15·6	0·3	34·3
A	24	1·66	1·30	0·84	3·22	2·67	3·61	10·63	1·09	18·7	13·7	0·4	32·8
Control													
P	24	0·00	0·01	0·00	0·17	—	0·75	0·93	0·74	—	—	—	—
A	24	0·01	0·00	0·02	0·19	—	0·75	0·97	0·74	—	—	—	—

The results of all four experiments are presented graphically in Figs. 2 and 3. In Fig. 2 are compared the losses of sugar from the external solutions and the sums of the gains of sugar and starch by the disks, together with the CO_2 output, all calculated as hexose. The data provide duplicated series for sucrose and invert sugar feeding, similar in all respects except labelling. Losses from the external solutions and overall gains by the disks proceeded in a virtually linear manner throughout the 24-hour periods of the experiments. The lines drawn in Fig. 2 are accordingly calculated regression lines.

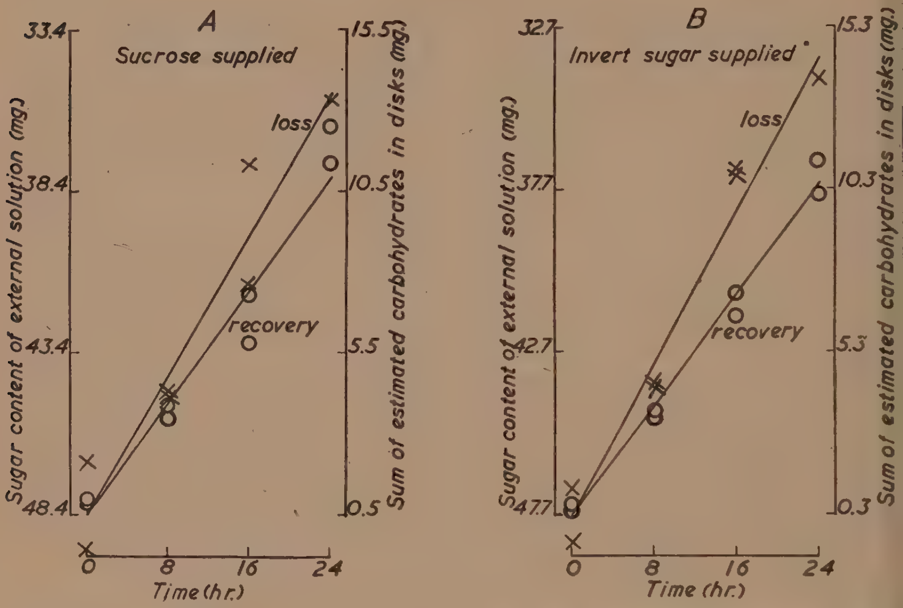

FIG. 2. Sugar content of external solutions and the sums of carbohydrate fractions and of CO_2 evolved (all calculated as hexose) in tobacco-leaf disks floated on sucrose (*A*) and invert sugar (*B*) in an atmosphere of oxygen at 25° C. for 24 hours. The lines are the linear regressions of carbohydrate on time, each calculated from duplicate experiments (8 observations).

Sugar disappearing from the external solutions (loss) —X—. Components estimated in lea disks (recovery) —O—.

The regression coefficients representing rates of loss from the external solution or gain by the disks in mg./8 hours were:

Sucrose supplied . . .	loss from solution 4·28,	gain by disks 3·53
Invert sugar supplied . . .	,, ,, ,, 4·67,	,, ,, ,, 3·34

It is evident that loss from the external solutions and overall gains by the disks are the same whichever type of sugar was supplied. There is, however a significant difference between the rates of loss and gain, and it must be concluded that the estimated components only account for about 75 per cent of the sugar taken up. The nature of the products formed by the remainin 25 per cent. of sugar has not yet been ascertained. Although the total rate o loss from the external solutions was independent of the type of sugar present when invert sugar was supplied glucose disappeared at a faster rate tha fructose (see Table III). Calculation of the ratio of glucose to fructose lost from the estimates of the amounts in the external solutions, gave a value o 1·66. This figure is only approximate, since differences between initial an final hexose contents from which it is derived are obviously subject to rather

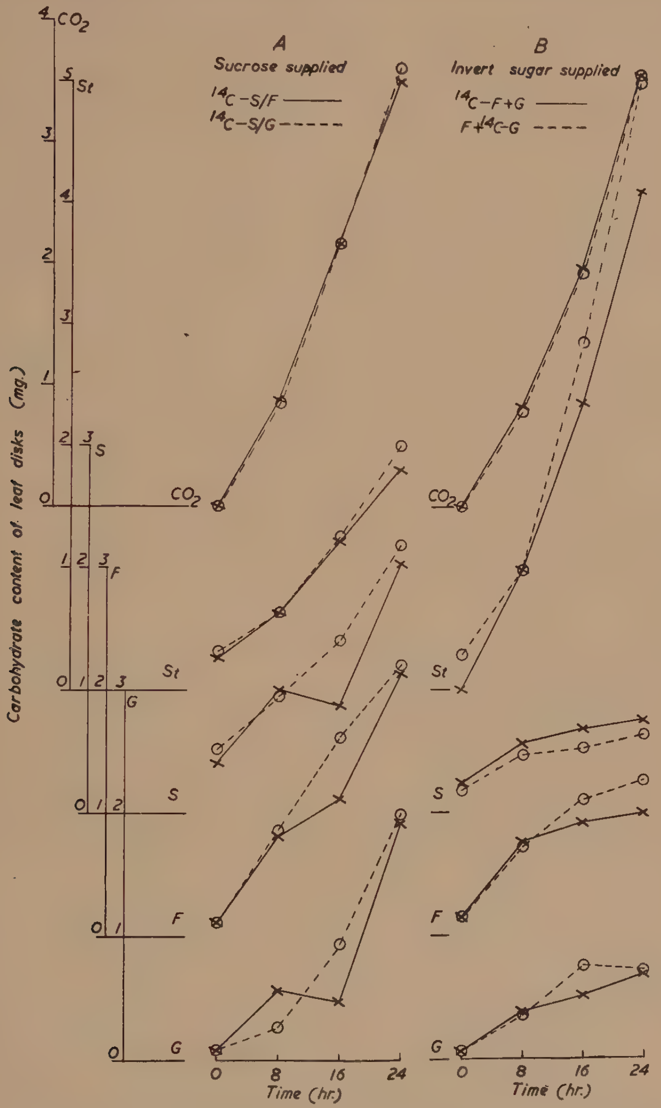

FIG. 3. Estimates of carbohydrate fractions and of CO_2 evolved (all calculated as hexose) in tobacco-leaf disks floated on sucrose (*A*) and invert sugar (*B*) in an atmosphere of oxygen for 24 hours at 25° C. At time 0 when the first CO_2 measurement was made, the disks had been in contact with the sugar solutions for about 1 hour. *G*, glucose; *F*, fructose; *S*, sucrose; *St*, starch; CO_2, carbon dioxide evolved, in mg. per 12 disks.

arge errors. However, it may be concluded that when the leaf disks were loated on invert sugar unequal amounts of glucose and fructose acted as ubstrate for further metabolism.

In Fig. 3 are shown changes in the estimated components of the leaf disks together with the CO_2 output. When the disks were floated on sucrose, increase in the components proceeded at a nearly constant rate throughout the 24-hour period of the experiment. When floated on invert sugar, however, the rate of sugar accumulation fell off after the first 8 hours and there was a corresponding increase in the rate of starch accumulation. For purposes of comparison mean rates of increase over 24 hours have been calculated assuming a linear relationship with time. The calculated rates are shown in Table IV. CO_2 output constituted 30 per cent. of the total of estimated components whichever sugar was supplied. Where sucrose was fed, sugars and starch accumulated at approximately equal rates, each contributing about 17 per cent. of the total. When invert sugar was fed there was a much larger accumulation of starch which here constituted 50 per cent. of the total, the three sugars taken together contributing only 20 per cent. The most striking difference between the results of sucrose or invert sugar feeding was therefore the large difference in the starch/sugar ratio in the disks. Although in these experiments invert sugar appeared to be more readily available for starch synthesis than sucrose, it must be remembered that the opposite claim has frequently been made (for example, Parkin, 1899). Further data are required before the difference obtained here can be considered to hold in general.

TABLE IV

Mean rates of increase of sugars and starch in tobacco-leaf disks floated on 5 per cent. sucrose or invert sugar, and rate of carbon dioxide evolution (conditions as detailed for Table III). Estimates are the linear regression coefficients of the components on time. (Fig. 3)

Leaf component	Floated on: Sucrose (mg./12 disks/8 hrs.)	Floated on: Invert sugar (mg./12 disks/8 hrs.)
Fructose	0·66	0·32
Glucose	0·59	0·22
Sucrose	0·51	0·16
Starch	0·54	1·49
Carbon dioxide	1·20	1·20
Total	3·50	3·39

Respiratory substrate

The respiratory quotient when leaf disks were floated on 5 per cent. sugar solutions was 1·07 to 1·10, but when floated on water the R.Q. of the endogenous respiration was 0·75 (see Table III) and this rate was maintained for at least 24 hours. If it be assumed that the endogenous respiration continued undiminished in disks supplied with sugar solutions, the R.Q. of the extra respiration due to sugar can be calculated by subtracting the values found for oxygen uptake and CO_2 output in the absence of sugar. Such calculations lead to an estimate of the R.Q. for the sugar respiration of 1·25, which would imply considerable aerobic fermentation of some sort. It seems unlikely that sufficient fermentation could occur to account for so high a value as 1·25, and it is therefore concluded that the endogenous respiration is largely suppressed by adding sugar. This conclusion receives support from the fact that the relative specific activity of the CO_2 evolved was equal or nearly equal to that of the sugar entering the disks (see Table V). During sugar respiration, therefore, dilution of the CO_2 by oxidation of unlabelled material must have been negligible. It should, however, be noted that Cochrane and Gibbs (1950) obtained an opposite result working with *Streptomyces coelicolor*.

Radioactivity in the external solutions

The relative specific activity of the sugars in the external solutions remained constant during the 24 hours of the experiments, and no transfer to the unlabelled moeity either in sucrose or invert sugar was detected.

Distribution of activity in the components of the leaf disks

Relative specific activities of starch and sucrose and of CO_2 evolved during sugar feeding of the leaf disks are shown in Table V together with estimates

TABLE V

Relative specific activities of sucrose and starch isolated from tobacco-leaf disks floating on 5 per cent. solutions of radioactive sugars, and of the carbon dioxide evolved. Conditions as described on p. 50

	On sucrose				On invert sugar			
Time (hrs.)	Sugar supplied	Constituents of leaf disks			Sugar supplied	Constituents of leaf disks		
		Sucrose	Starch	CO_2		Sucrose	Starch	CO_2
		c.p.m./10^{-3} mg. (components calculated as hexose)						
	S/^{14}C–F				^{14}C–F+G			
8	8·6	8·4	5·5	8·1	9·0 (6·7)*	6·1	6·8	6·6
16	7·6	9·1	7·7	7·7	9·7 (7·3)*	6·6	8·5	7·8
24	8·4	6·4	8·3	7·7	9·7 (7·3)*	8·0	8·5	8·6
Mean	8·2	8·0	7·2	7·8	9·5 (7·1)*	6·9	7·9	7·7
	S.D. of means ±0·61				S.D. of means ±0·54			
	S/^{14}C–G				F+^{14}C–G			
8	7·9	9·4	7·7	9·6	8·9 (11·2)*	13·4	9·4	9·1
16	8·6	8·7	10·4	8·9	9·4 (11·8)*	13·6	10·6	9·9
24	8·4	6·7	11·0	9·1	9·2 (11·5)*	12·9	10·6	9·9
Mean	8·3	8·3	9·7	9·2	9·2 (11·5)*	13·3	10·2	9·6
	S.D. of means ±0·66				S.D. of means ±0·28			

* Calculated on the assumption that the ratio of glucose to fructose entering the disks was 1·66; see text.

of activity in the external solution. The specific activities of sucrose and CO_2 did not change significantly with time of exposure of the disks to sugar, but those for starch rose a little. This is probably because little starch was formed during the first 4 to 6 hours so that at the first estimation after 8 hours, dilution by the small amount of inactive starch present initially was measurable. In general the uniformity of the results indicates that a steady state is established between rates of entry of sugar and redistribution of the labelling between leaf components.

Inspection of Table V shows that when sucrose was supplied whichever moiety was labelled the mean values for relative specific activities of sucrose, starch, and CO_2 from the disks were equal and the same as that of the entering sucrose.

When invert sugar was supplied the mean relative specific activities of starch and CO_2 were again equal, but the relationship to the activity of the entering sugar is less certain owing to the unequal uptake of glucose and fructose (see p. 52). The activity of the invert sugar supplied was less than that of starch and CO_2 when glucose was the initially labelled hexose and greater when fructose was initially labelled. The excess of glucose entering the leaf disks thus resulted in utilization of more glucose than fructose in further metabolism and it appears that the hexose was used in relation to the amount presented, rather than to the nature of the hexose. Estimates of the activity of the entering sugar, calculated using the ratio of glucose to fructose of 1·66 (see p. 52), are shown in parentheses in Table V. Where fructose was labelled, the adjusted figures are equal to those for sucrose, starch, and CO_2

TABLE VI

Relative specific activities of free hexoses and of hexoses combined in sucrose, isolated from tobacco-leaf disks floating on 5 per cent. solutions of radioactive sugars. Conditions as described on p. 50

	On sucrose					On invert sugar				
Time (hrs.)	Sugar supplied	Constituents of leaf disks				Sugar supplied	Constituents of leaf disks			
		Free		Combined			Free		Combined	
		Fructose	Glucose	Fructose	Glucose		Fructose	Glucose	Fructose	Glucose
				c.p.m./10^{-3} mg. (sucrose moieties calculated as hexose)						
	S/^{14}C–F					^{14}C–F+G				
8	8·6	21·6	1·4	24·0	0·8	9·0 (6·7)*	9·5	4·3	7·7	4·5
16	7·6	20·2	3·1	25·5	0·9	9·7 (7·3)*	10·8	5·3	8·9	5·3
24	8·4	19·5	1·1	17·3	0·9	9·7 (7·3)*	12·7	5·3	9·5	6·1
Mean	8·2	20·4	1·9	22·3	0·9	9·5 (7·1)*	11·0	5·0	8·7	5·3
	S/^{14}C–G					F+^{14}C–G				
8	7·9	3·4	18·3	2·2	19·5	8·9 (11·2)*	9·5	16·6	11·7	10·4
16	8·6	2·4	19·5	4·4	18·8	9·4 (11·8)*	10·0	16·2	9·2	12·4
24	8·4	1·7	16·1	2·0	15·1	9·2 (11·5)*	9·8	17·0	11·0	12·2
Mean	8·3	2·5	18·0	2·9	17·8	9·2 (11·5)*	9·8	16·6	10·3	11·7

* Calculated on the assumption that the ratio of glucose to fructose entering the disks was 1·66; see text.

from the leaf disks. Where glucose was labelled they are higher than those for starch and CO_2 but less than those for sucrose. Independent estimates of the activity of sucrose made from measured values for the component hexoses (see Table VI) gave a mean value of 11·2, so that possibly in this instance an unrecognized systematic error may account for the rather high values obtained in the direct measurement of sucrose activity. It is considered likely that with invert sugar feeding as well as with sucrose the activity of the starch, sucrose, and CO_2 was equal, or nearly so, to that of the entering sugar, but this conclusion must remain tentative until more data are available.

The specific activities of free hexoses in the disks and of the moieties of sucrose are presented in Table VI. Where sucrose was supplied the asymmetry of label was retained, only traces of activity appearing in the hexose residue initially inactive. Free hexoses had the same asymmetric labelling as the sucrose, from which it appears that sucrose which is not at once metabolized accumulates in the cell, and gives rise to hexoses by inversion, which latter do not become involved in any interconversion system. It must be emphasized that this asymmetry of label persisted for long periods of time, in this case for at least 24 hours. When invert sugar was fed, the sucrose, which here resulted from synthesis, was strongly labelled in both moieties. The ratio of activities of the hexose components was when fructose was initially the labelled hexose $F/G = 1{\cdot}6/1{\cdot}0$ and when glucose was initially labelled $0{\cdot}9/1{\cdot}0$. The hexose residues were therefore probably not quite equally labelled, but there must in any case have been considerable interchange of activity between the fructose and glucose structures prior to sucrose synthesis. The free hexoses were also strongly but unequally labelled. Whichever hexose was initially labelled the corresponding sugar in the disk was less labelled than that in the external solution, but still had about twice the activity of the initially unlabelled hexose.

DISCUSSION

When starch synthesis is induced by floating leaf disks on sugar solutions in the dark, or in weak light, it is accompanied by a high CO_2 output and by high internal sugar concentrations and the rate of synthesis is slow compared with that from CO_2 in the light. The amount of starch formed depends upon such factors as temperature, oxygen tension, and the type and concentration of sugar supplied. As yet little information is available as to how synthesis varies with these factors, and in the present experiments they have been maintained at arbitrarily chosen levels. No claim is made that the conditions of temperature 25° C., an atmosphere of oxygen, and an external sugar concentration of 5 per cent. are optimal for starch synthesis.

The distribution of starch in leaf pieces shows that sugar enters most readily through the vascular bundles and more slowly at the cut edges of the tissue, from whence it spreads slowly inwards (Parkin, 1899; Waters, 1928). In tobacco leaf disks starch could be detected by its iodine stain after about 4 hours' exposure to 5 per cent. sugar solutions, but increase in respiration was evident after about an hour, and the rate continued to increase for about 6 hours as the sugar penetrated across the disks. Saturation of the respiratory system thus appeared to precede starch formation (see p. 53). A steady state was reached after about 8 hours, and at this stage rates of starch formation and CO_2 evolution became constant. As soon as sugar entered the disk it

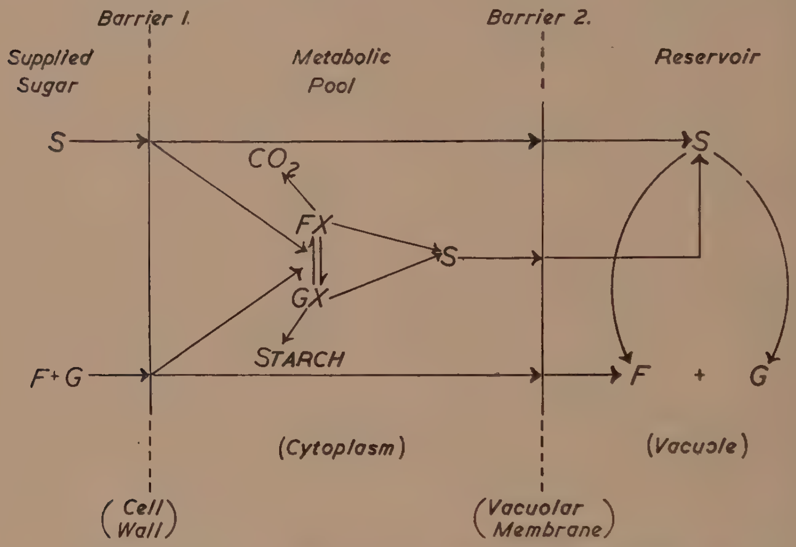

FIG. 4. Scheme illustrating carbohydrate transformations in tobacco-leaf disks floating on 5% sucrose or invert sugar in an atmosphere of oxygen at 25° C. *S*, sucrose; *F* and *G*, fructose and glucose; *FX* and *GX*, derivatives of fructose and glucose, possibly phosphate esters. See text.

probably became the sole respiratory substrate as evidenced by the rise in R.Q. to values slightly over one and the similar specific activities of the CO_2 evolved and the sugar supplied (see p. 54).

Under the conditions employed sucrose probably entered the cell unchanged. It has been claimed that inversion precedes uptake because reducing sugar appears in the external medium when tissues are floated on sucrose solutions (Said, 1948). Rapid production of reducing sugar also follows injection of sucrose into leaves (Ranjan quoted by James, 1953; Putman quoted by Stumpf, 1952) and these authors suggested that inversion occurs in the intercellular spaces. Weatherley (1953), on the other hand, found no relation between the amount of reducing sugar appearing in the external solution and amount of sugar taken up and concluded that sucrose enters intact. When asymmetrically labelled sucrose was supplied in the present experiments the sucrose accumulating in the disks retained its initial labelling, indicating that it entered intact. When asymmetrically labelled invert sugar was supplied, however, the sucrose synthesized was strongly labelled in both moieties, and also more starch was formed. These differences between the effects of sucrose and invert sugar feeding should not occur if sucrose was inverted prior to uptake. Furthermore, since glucose is taken up more rapidly than fructose (see p. 52), extracellular inversion should result in an excess of fructose in the external medium, and no such excess was found. This evidence therefore favours the view that sucrose enters the cell as such, and although the data support the suggestion that inversion takes place at a site remote from that of synthesis and respiration they do not indicate that the site is extracellular (see below).

A tentative scheme to accommodate the results so far obtained is presented in Fig. 4. It is an elaboration of one based on some preliminary experiments of the same type (Porter, 1953). It is postulated that sugar enters the cell across a barrier into a site of metabolic activity, and then after crossing a second barrier emerges into a reservoir where little metabolism can occur. As a speculation the first barrier might be identified with the cell surface adjacent to the cellulose wall or with specialized regions in this surface. The active metabolic site might be the cytoplasm, or the cytoplasmic inclusions, the second barrier the vacuolar membrane, and the reservoir the vacuole. It is supposed that a proportion of the sugar entering the cell is at once incorporated into a metabolic pool, but with the concentration of sugar used here the rate of uptake from the external solution is greater than the rate of incorporation into the pool, so that some sugar passes across into the vacuole, where it accumulates. It is further supposed that uptake into the pool involves the immediate formation of glucose and fructose derivatives which are rapidly interconvertible, and that the mechanism of incorporation of sucrose must provide for simultaneous formation of derivatives of both hexose components. From the common source so created CO_2 and starch are formed when sucrose is the substrate, and also sucrose when invert sugar is the substrate. In this way account is taken of the observed similarity between the specific activities of starch, CO_2, and that of the supplied sugar as well as the nearly equal labelling in the moieties of sucrose synthesized from hexose. When invert sugar was the substrate the distribution of activity was consistent with incorporation of the hexose presented as a whole, irrespective of the relative amounts of glucose and fructose (Table V). Thus when glucose was the labelled hexose the specific activities of starch and CO_2 were higher than when fructose was labelled, in accordance with the fact that more rapid entry of glucose leads to more glucose than fructose reaching the metabolic site.

Calvin and his collaborators (Benson and Calvin, 1950) have shown that sugar phosphates arise before free sugars during photosynthesis, and Putman (loc. cit.) has recorded the transient formation of phosphate esters during sugar-feeding experiments, so that these esters may well be the hexose derivatives which undergo interconversion. If this should prove to be the case it would mean that fructose and glucose are equally readily phosphorylated by the leaf system and further that phosphorylation might be the step requiring oxygen in the synthesis of starch from free sugar. In photosynthesis the sugar phosphates do not derive from free hexose, so that formation of such substances by a path not normally utilized may perhaps be a rate limiting step and account for the slow rate of formation of starch when leaf disks are floated on sugar solutions as compared with formation from CO_2 in the light.

To account for the fact that total uptake of sugar was the same from sucrose and invert sugar, yet 50 per cent. of the uptake accumulated as sugar and only 17 per cent. as starch from sucrose, while from invert sugar only 20 per cent. appeared as sugar, and 50 per cent. as starch, it may be supposed that sucrose is incorporated into the pool less rapidly than invert sugar. This view is supported by the fact that sucrose accumulating in the disks retained its asymmetry of label and that there was no exchange of label between free hexoses produced from it. The sucrose not at once taken into the pool is presumed to cross into the vacuole unchanged and there become partially inverted. As the nature of the supplied sugar did not affect the CO_2 output it is concluded that withdrawal of material from the common source to meet respiration requirements takes precedence over withdrawal for starch synthesis. In these circumstances if conversion of sucrose to the common source is rate-limiting as far as starch formation goes, no excess of equilibrated material will be available for new sucrose synthesis, in which both components will be labelled, and in consequence only unchanged sucrose reached the vacuole (Table VI).

The greater amount of hexose incorporated into the pool may be presumed to saturate both respiratory and starch-forming mechanisms and excess material is then diverted to sucrose synthesis in which both moieties will be equally or nearly equally labelled. If such sucrose is discharged into the vacuole and there is partly inverted, the hexoses will mix with free hexose which has not become incorporated into the pool and so remains asymmetrically labelled; the resulting mixture will then contain fructose and glucose both strongly but unequally labelled (Table VI).

On the basis of these experiments it would not be necessary to assume re-entry of sugar into the pool from the vacuole. However, in preliminary experiments carried on for periods up to 72 hours (Porter, 1953) appreciable activity was found in the moiety of sucrose not initially labelled as well as in the corresponding free hexose. On the present scheme, this could only occur if there were, at least after a time, a slow movement of sugar from the vacuole back into the metabolic pool.

It must be concluded from the data that fructose and glucose, whether supplied free or combined as sucrose, were equally available for starch synthesis and CO_2 production, and there can be no question of preferential utilization of one or other hexose. It would also appear that if sucrose is the substrate the glycoside link must be broken to allow for interconversion of the hexose components and therefore free sucrose cannot act as a precursor for starch synthesis by a transfer mechanism such as for example amylosucrase. Similarly, free hexose does not seem to be a direct precursor of sucrose, since the equality of labelling in the sucrose synthesized by the cell from material in which only one hexose was labelled demands that sucrose shall arise from the common source of hexose derivatives. A mechanism involving sucrose phosphorylase is thus unlikely.

Since this work was completed Turner (1953) and Leloir and Cardini (1953) have achieved the enzymic synthesis of sucrose *in vitro*, using enzyme preparations derived from plant sources. Turner's data also led him to state that sucrose phosphorylase was probably not the mechanism. Leloir and Cardini's enzyme effects synthesis from uridine diphosphoglucose and free fructose and in their view no phosphorylated intermediate of fructose is involved. Since free fructose in the cell does not appear to be the source of the fructose moiety of sucrose, the way in which this enzyme might operate *in vivo* is not yet clear, and attention is focused on the question as to how both moieties of sucrose can be simultaneously converted to the postulated hexose derivatives, and how sucrose is produced therefrom.

It is recognized that the particular conditions under which these experiments were performed may have led to results some of which are not of general application. However, since this paper was written the detailed results of Putman's (loc. cit.) work have been published (Putman and Hassid, 1954), and also the work of Vittorio, Krotkov, and Reed (1954). Putman and Hassid infiltrated leaf disks of canna with low concentrations of radioactive glucose or fructose, so that the sugar content of the leaves was not appreciably altered, and examined the redistribution of radioactivity over periods up to 360 minutes. They report synthesis of sucrose from each hexose, in which the sucrose moieties were nearly equally labelled, and no activity was found in the free hexose not supplied as substrate. Vittorio *et al.* kept tobacco leaves with their petioles in 0·2 M. radioactive glucose solution for 6 hours, and they also found that sucrose was synthesized nearly equally labelled in both moieties, while free fructose did not become active. Under three quite different experimental conditions, therefore, similar results have been obtained with respect to sucrose synthesis and the labelling of free hexose. The concept of a system which rapidly equilibrates derivatives of glucose and fructose and provides a pool from which material is withdrawn for further metabolic events thus may be generally true. All the results also support the notions that inversion of sucrose takes place at a site remote from that of active metabolism, and that free hexoses are not direct precursors of sucrose. It is manifest that final elucidation of the way in which glucose and fructose are interconverted remains an urgent problem.

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A Survey of the Rates and Products of Short-term Photosynthesis in Plants of Nine Phyla[1]

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SUMMARY

1. Short-term photosynthetic experiments using $C^{14}O_2$ and paper chromatography were performed with 27 different plants representing nine phyla: Schizophyta (Schizophyceae), Euglenophyta, Chlorophyta, Charophyta, Chrysophyta, Rhodophyta, Bryophyta, Pteridophyta, and Spermatophyta.

2. There is a remarkable uniformity in the types of ethanol-soluble compounds which became radioactive in the entire group of plants used. The amounts of the different compounds varied considerably percentagewise among the various plants as would be expected because of their inherent metabolic differences and the variations in their physiological states induced by experimental conditions.

3. Sucrose became radioactive in very different amounts in two major groupings of plants: (*a*) those containing only photosynthetic tissue, and (*b*) those containing non-photosynthetic tissue as well. The amount of radioactive sucrose in the former group was much lower than that in the latter.

4. An unidentified compound became radioactive in appreciable amounts in two of the blue-green algae, but was radioactive in very small amounts or not visible at all on the chromatograms of all other plants.

INTRODUCTION

In consideration of the great differences in general physiology among plants the question arises whether there is a uniformity in the reactions involved in the fixation of carbon during the early steps of photosynthesis or whether there may also be differences in these basic reactions. To investigate this question and to determine whether any differences, should they occur, might be correlated with phylogenetic relationships, a survey of representatives of major plant groups was made with respect to the kinds and relative amounts of the compounds into which carbon was incorporated during short-term photosynthesis. The general methods employed in the study were tracer

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technique using C^{14} for labelling compounds and paper chromatography for separating and identifying them.

EXPERIMENTAL METHODS

Photosynthesis experiments were performed with the following plants: *Phormidium*, *Synechococcus*, and two species of *Nostoc* (blue-greens); *Chlorella*, *Scenedesmus*, *Haematococcus*, *Spirogyra*, and *Chlorococcum* (green algae); *Nitella*; *Vaucheria* (a yellow-green alga); *Euglena*; *Porphyridium* (a red alga); *Fontinalis* and *Funaria* (mosses); *Conocephalum* (a liverwort); *Ceratopteris* and *Polystichum* (ferns); yew, juniper, squash, soybean, *Kalanchoë*, tomato, ivy, avocado, and barley.

With the exception of *Haematococcus*, *Spirogyra*, *Nitella*, yew, juniper, and ivy, all of which were used directly from their places of growth, the plant materials used in these experiments were grown in the laboratory. The algae were grown under conditions shown in Table I and in media described in Table II. The mosses, liverwort, and ferns were grown in dishes of either pond water or V medium on a fluorescent light shelf. The squash, soybean, *Kalanchoë*, tomato, and avocado were grown in a fluorescent light chamber.

TABLE I

Culturing conditions for algae

Algae	Culture vessel	Aeration	Agitation	Medium	Light
Phormidium	250-ml. Erlenmeyer	No special provision	Occasional swirling by hand	V	Fluorescent
Nostoc muscorum	2,500-ml. culture flask	Air bubbled through	,,	PG	,,
Nostoc sp.	250-ml. Erlenmeyer	No special provision	,,	Soil and water	,,
Synechococcus cedrorum	2,500-ml. culture flask	Air drawn over	Constant shaking	PG+0·1% Na glutamate	Incandescent
Chlorococcum sp.	2,500-ml. culture flask	,,	,,	V	Fluorescent
Chlorella	,,	,,	,,	,,	,,
Scenedesmus	,,	,,	,,	,,	,,
Euglena	,,	No special provision	Occasional swirling by hand	E	,,
Vaucheria	250-ml. Erlenmeyer	,,	,,	V	,,
Porphyridium	2,500-ml. culture flask	Air drawn over	Constant shaking	S and B	Incandescent

Because of the differences in form between the algae and the other plants, two general methods of carrying out the CO_2 fixation experiments were employed. The algae were centrifuged from their nutrient media, washed once with distilled water, and centrifuged again. 75 c.c. of a 1 per cent. suspension of algae in distilled water (based on packed cell volume) was poured into a flat chamber (Fig. 1). This chamber was illuminated between two incandescent lamps providing light intensities shown in Table III for a 15- or 20-minute period to permit the algae to adjust to the light conditions. The suspension was kept at about room temperature during this period by placing the chamber in a water-cooled bath. Throughout this period and the rest of the experiment heat from the lamps was reduced by means of water-cooled infra-red heat filters placed between the lamps and the illumination chamber. Following

TABLE II

Contents of media used for algae

	V	PG	E	S and B
Cation-free water	1 l.	1 l.	1 l.	500 c.c.
Sea water	—	—	—	500 c.c.
1 M. KNO_3	5 c.c.	11·7 c.c.	—	5 c.c.
1 M. K_2HPO_4	0·5 c.c.	5·7 c.c.	3 c.c.	0·6 c.c.
1 M. KH_2PO_4	0·5 c.c.	—	—	—
1 M. $MgSO_4.7H_2O$	2 c.c.	1 c.c.	2 c.c.	0·2 c.c.
0·1 M. $Ca(NO_3)_2$	0·25 c.c.	1 c.c.	—	—
1 M. NH_4NO_3	—	—	6 c.c.	—
1 M. NH_4Cl	—	1 c.c.	—	—
$NaAc.3H_2O$	—	—	5 g.	—
Difco tryptone	—	—	5 g.	—
Trace elements solution*	1 c.c.	1 c.c.	—	—
Iron and EDTA solution†	1 c.c.	1 c.c.	1 c.c.	1 c.c.
Adjusted to pH	~6·8	~8·5	~7·0	~8·5

* 1-c.c. trace elements contains 1·43 mg. H_3BO_3, 1·05 mg. $MnSO_4 \cdot H_2O$, 0·05 mg. $ZnCl_2$, 0·04 mg. $CuSO_4 \cdot 5H_2O$, 0·01 mg. $H_2MoO_4 \cdot H_2O$.

† The iron and ethylenediamine-tetracetic acid solution was prepared according to the method described by Jacobsen (1951). The concentration of $FeSO_4 \cdot 7H_2O$ in media with 1 c.c. of this solution per litre is 0·0009 M.

the adjustment period 200 μl. of sodium bicarbonate solution containing 400 μc. C^{14} per ml. was injected into the vessel, which was stoppered immediately and shaken continuously in the light beam for 5 minutes. At this time the contents were dumped into 300 c.c. of boiling absolute ethanol, thus making an 80 per cent. ethanol solution. This mixture was then brought to a boil again and immediately cooled in a cold-water bath.

FIG. 1. Apparatus for alga experiments

The total amount of activity fixed by the algae was determined by drying an aliquot of the ethanol suspension on an aluminium plate and counting the radioactivity with a wide-mouth Geiger–Müller counter. The amount of activity fixed in 80 per cent.-ethanol soluble compounds was determined by centrifuging the ethanol suspension and similarly plating and counting an aliquot of the supernatant. The distribution of activity in the various compounds in the 80 per cent.-ethanol extract was determined by two-dimensional chromatography of a concentrate of the extract on Whatman No. 1 paper.

Phenol-water was used as the first solvent and a butanol-propionic acid-water mixture as the second solvent. Radioautograms of the resulting chromatograms were made with 'No-Screen' X-ray film in order to find the location of the radioactive compounds. These compounds were identified by the relation of their positions to known compounds and to each other and, in cases of doubt, by co-chromatography with a known compound (Benson *et al.*, 1950). The activity in each spot on the chromatograms was counted and tabulated in terms of percentage of the total activity on the chromatogram.

The CO_2 fixation experiments with the higher plants were carried out in a gas-tight chamber with parallel glass sides (Fig. 2). The thalli, leaves, or larger portions of plants used for the experiments were transferred to small tubes of water in this chamber as quickly as possible after being removed from the rest of the plant. Thalli or leaves which appeared likely to wilt during the experiments were sprayed with water prior to the adjustment period. The chamber was illuminated in the same way as it was for the algae with the varying light intensities shown in Table III. The chamber was closed and air drawn through it during the adjustment period which varied from 5 to 15 minutes. Following this time the chamber was partially evacuated and radioactive CO_2 was introduced in the amounts shown in Table III. After 5 minutes' exposure to radioactive CO_2 (10 minutes in the case of the liverwort) the plant parts were removed either directly into boiling 80 per cent. ethanol in the case of both ferns, *Funaria*, squash, soybean, tomato, and barley or first into liquid nitrogen, where they were ground, and then into boiling 80 per cent. ethanol in the case of *Fontinalis*, liverwort, yew, juniper, *Kalanchoë*, ivy, and avocado. The total activity, the activity in the 80 per cent.-soluble portion and its distribution in the various compounds was determined in the same way for these suspensions as it was for the corresponding suspensions of algae.

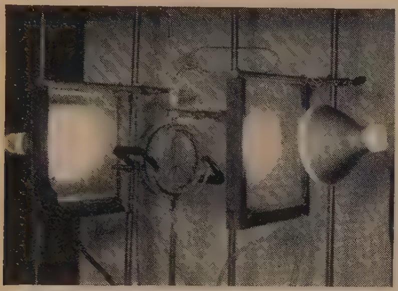

FIG. 2. Apparatus for leaf experiments

RESULTS

The rates of CO_2 fixation of the 19 plants tested varied greatly as might be expected with such a wide diversity of plant types, ages of the material, &c. The rates ranged from 0·3 μ mol. CO_2 fixed/g. of wet plant material in

5 minutes in the case of the liverwort to 14·5 μmol./g./5 min. in the case of *Fontinalis*. (See Table III.) There was also a wide variation in the percentage of the fixed activity which was extractable with 80 per cent. ethanol. For those plants in which experimental methods permitted this value to be obtained, this figure varied from 17·7 per cent. for *Kalanchoë* to 88·8 per cent. for yew.

Although there are wide variations in the rates at which the different plants fixed CO_2 and incorporated it into insoluble materials, there was a remarkable similarity in the kinds of soluble compounds formed. However, their percentagewise distribution showed great variation. The detailed results are reported in Table III and some representative chromatograms from which the data were obtained are shown in Fig. 3.

DISCUSSION

Several striking points stand out in the results of this survey of the photosynthetic pattern of a variety of plants. One of these is the remarkable uniformity with which the various phosphates and amino-acids are found to become radioactive in this type of experiment. Hexose monophosphates appeared radioactive on chromatograms of all 19 plants, while pentose- and triose-phosphates and phosphoglyceric acid were found in all but one. The uridine-diphosphate-glucose and UDP-galactose spot was on all but 3 papers, and ribulose diphosphate, phosphoglycolic acid, and phospho-enolpyruvate were easily visible on the majority of chromatograms. Of the amino-acids, serine and alanine became radioactive in all the experiments, aspartic and glutamic acids in 17 and glycine in 16. It is quite probable that compounds which appeared radioactive in all but a few of the experiments as described actually were radioactive in every one but to such a low degree that the activity was not detectable by the procedures used in these experiments. The general uniformity in the compounds which became radioactive in these varied plants indicates that the same basic pattern of reactions occurred in the incorporation of CO_2 into cell constituents in all of the plants tested. And since nearly all major plant phyla are represented in the experiment it seems safe to assume that this set of reactions is the one which is responsible for all photosynthetic CO_2 fixation and early metabolism.

Despite the uniformity in the occurrence of these compounds, there is a great variation in the percentagewise distribution of radioactivity among the various plants. Individual variations cannot yet be interpreted since they certainly must be expected in a survey of this type for a number of reasons, residing partly in the experimental procedures and partly in the organisms themselves. Conditions under which the experiments were performed were patterned after those previously found to be suitable for *Scenedesmus* and soybean and were certainly not optimum for all, if for any, of the organisms tested. In some of the organisms these conditions almost certainly induced quite abnormal physiological states. In addition to the variation caused by

TABLE III

Radioactive CO_2 fixed in 5-minute photosynthesis experiments

	Phormidium	*Nostoc muscorum*	*Nostoc* sp.	*Synechococcus*	*Haematococcus*	*Chlorococcum*	*Chlorella*	*Scenedesmus*	*Spirogyra*	*Nitella*	*Euglena*	*Vaucheria*	*Porphyridium*	*Funaria* (protonemata)	*Fontinalis* (mature gametophyte)	Liverwort* (mature gametophyte)	*Ceratopteris* (mature sporophyte)	*Polystichum* (prothalli)
Condition of experiment																		
Age of plant material	20 days	20 days	—	4 days		48 hrs.	3 days	24 hrs.	—	—	6 days	—	19 days	20 days	—	—	—	20 days
Light intensity (foot-candles on each side of lollipop)	2,700	6,500	2,700	—	2,700	—	30,000	7,500	2,700	2,700	7,500	2,700	7,500	2,720	2,000	900	600	2,700
CO_2 given (μc.)	100	80	100	80	40	—	150	80	100	100	80	100	80	100	253	500	227	100
Specific activity of CO_2 (mc./mMol $BaCO_3$)	16·1	16·1	—	16·1	16·1	—	8·0	16·1	16·1	16·1	16·1	16·1	16·1	16·1	9·1	9·1	9·1	16·1
Activity fixed																		
Total counts fixed in 5 min./c.c. packed algae or g. wet wt. leaves (10^6 c./min.)	—	3·0	—	2·0	—	—	26·4	15·5	—	—	2·7	—	1·9	—	42·0	0·9	—	—
Counts fixed in 80% ethanol-soluble compounds (10^6 c./min.)	2·8	2·5	0·8	0·6	1·3	—	—	10·0	6·4	4·0	1·5	10·4	0·5	17·8	28·0	0·7	0·8	1·6
% of fixed activity in solubles	—	83·3	—	28·8	—	—	—	64·1	—	—	54·7	—	24·3	—	66·5	77·8	—	—
Distribution of activity in 80% ethanol-soluble compounds (% of total on chromatogram)																		
Phosphates																		
Ribulose diphosphate	—	5·5	2·0?	20·8	—	15·1	2·8	2·5	1·4	5·0	3·3	5·3	14·9	t	t	5·2	?	—
Uridine-diphosphate-glucose and UDP-galactose	t	7·5	4·7	8·3	1·4	4·8	7·6	17·4	12·3	6·2	16·7	5·5	3·9	5·7	0·8	?	6·3	8·8
Hexose monophosphates	28·6	6·7	23·8	20·8	10·8	16·7	12·1		25·9	40·8	43·4	18·6	12·7	11·7	9·4	21·0	26·2	17·0
Phosphoglyceric acid	21·8	2·5	35·7	16·8	0·7	10·2	25·0	4·5	7·5	24·0	10·4	36·4	5·2	11·6	2·2	2·9	5·7	9·7
Phosphoglycolic acid	8·9	0·1	3·1	2·1	—	0·7	t	0·4	t	t	t	t	t			t	?	0·6
Phospho-enol-pyruvic acid	0·9	t		2·8	0·4	1·5	1·9	0·6	0·9	2·0	0·4	2·2	t	1·1	0·2	0·4	0·5	0·2
Pentose- and triose-phosphates	—	14·6	3·4	3·1	2·2	3·3	t	1·1	1·8	4·1	3·3	1·3	3·0	0·5	t	t	0·5	1·4
Unknowns in phosphate region	3·0	7·7	2·7	9·6	2·5	—	—	1·8	0·9	0·5	1·7	8·7	2·1	0·9	—	1·1	—	0·4

TABLE IIIa

	Yew	Juniper	Squash	Soybean	*Kalanchoë*	Tomato	Ivy	Avocado	Barley
Condition of experiment									
Age of plant material	Previous year's growth	Spring growth	4 weeks	5 weeks	16 weeks short day	Young leaves of 3 mths. plant	Previous year's growth	Young leaves of seedling	Seedling
Light intensity (foot-candles on each side of lollipop)	50,000	5,000	2,000	3,000	13,000	13,000	13,000	5,000	5,000
CO_2 given (μc.)	245	244	190	236	326	196	349	263	276
Specific activity of CO_2 (mc./mMol $BaCO_3$)	9·1	9·1	9·1	9·1	9·1	9·1	9·1	9·1	9·1
Activity fixed									
Total counts fixed in 5 min./c.c. packed algae or g. wet wt. leaves (10^6 c./min.)	1·6	—	—	—	1·7	—	3·1	—	—
Counts fixed in 80% ethanol-soluble compounds (10^6 c./min.)	1·4	2·5	15·2	37·0	0·3	10·8	2·6	10·1	11·5
% of fixed activity insolubles	88·8	—	—	—	17·7	—	83·9	—	—
Distribution of activity in 80% ethanol-soluble compounds (% of total on chromatogram)									
Phosphates									
Ribulose-diphosphate	—	0·6	?	0·1	—	t	—	3·3	1·4
Uridine-diphosphate-glucose and UDP-galactose	—	4·8	8·0	1·3	5·2	0·6	0·8	2·3	—
Hexose monophosphates	4·2	6·6	38·3	12·5	29·2	3·2	4·1	13·8	7·8
Phosphoglyceric acid	4·2	2·2	11·9	2·1	0·4	0·2	—	5·0	0·9
Phosphoglycolic acid	—	t	t	—	—	t	?	0·2	—
Phospho-enol-pyruvic acid	0·9	t	0·4	0·2	—	t	?	—	?
Pentose- and triose-phosphates	?	1·2	0·2	1·3	1·8	0·6	4·6	1·5	0·3
Unknowns in phosphate region	5·4	—	1·4	0·2	0·8	0·2	0·9	0·3	—

TABLE III*b*

	Phormidium	*Nostoc muscorum*	*Nostoc* sp.	*Synechococcus*	*Haematococcus*	*Chlorococcum*	*Chlorella*	*Scenedesmus*	*Spirogyra*	*Nitella*	*Euglena*	*Vaucheria*	*Porphyridium*	*Funaria* (protonemata)	*Fontinalis* (mature gametophyte)	Liverwort* (mature gametophyte)	*Ceratopteris* (mature sporophyte)	*Polystichum* (prothalli)
Sugars																		
Raffinose	—	—	—	—	?	—	—	—	—	—	—	—	—	—	5·2	—	—	—
Maltose	—	—	—	—	t	—	—	—	0·8	—	—	—	—	1·0	5·9	0·4	—	t
Sucrose	3·8	3·8	6·0	—	—	9·2	3·8	1·6	9·9	2·3	—	3·0	1·7	22·8	47·7	45·0	35·6	24·2
Glucose	2·3	2·5	—	—	—	—	1·1	—	—	—	0·5	—	—	—	—	—	2·1	—
Mannose, mannoheptulose, galactose, sedoheptulose	—	0·8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·5	—
Fructose	—	1·6?	?	—	1·1?	—	0·4	—	—	—	0·4	—	—	—	—	0·4	5·8	—
Ribulose	—	3·0	t	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Amino-acids																		
Aspartic acid	12·0	11·7	11·0	4·9	5·0	6·0	5·5	19·2	2·3	0·4	1·4	4·6	3·1	2·1	3·3	1·7	0·5	7·2
Glutamic acid	9·6	0·4	3·8	0·3	7·9	0·3	0·6	2·1	8·9	0·4	0·5	0·3	4·4	2·3	3·0	0·8	0·3	1·0?
Serine	t	1·0	t	1·5	2·5	6·7	7·1	6·9	1·7	1·1	2·5	4·1	7·9	6·5	1·6	0·8	0·5	2·9
Glycine	t	0·8	t	—	1·2	1·4	3·5	1·9	2·7	8·1	6·2	2·7	17·5	10·0	2·3	1·7	0·3	1·3
Threonine	—	—	—	—	t	—	—	1·7	—	—	—	—	—	—	—	—	—	—
Alanine	1·4	1·9	1·5	1·2	2·1	19·1	4·5	4·0	18·3	2·5	0·6	2·8	10·5	21·2	12·2	16·0	10·5	10·0
Misc. below alanine	—	—	—	—	19·6	—	—	—	—	—	—	—	3·0	—	—	—	—	—
Miscellaneous																		
Unknowns beyond alanine	—	—	—	—	—	—	—	9·3	—	—	—	—	6·0	—	—	—	—	—
Dextrins?	—	—	—	—	—	—	—	—	—	—	3·0	—	—	—	—	—	—	—
Unknown below alanine	2·6	20·9	0·9	6·7	t	—	—	0·2	0·5	—	0·4	—	1·3	0·6	—	0·4	—	—
Totals																		
In phosphate region	65·2	44·6	75·4	84·3	18·0	52·3	49·4	28·3	50·7	82·6	80·1	78·0	41·0	31·5	12·6	31·2	39·2	38·1
Sugars	6·1	11·7	6·0	—	1·1	9·2	5·3	1·6	10·7	2·3	0·9	3·0	1·7	23·8	58·8	45·8	44·0	24·2
Amino-acids	23·0	15·8	16·3	7·9	18·7	33·5	21·2	35·8	33·9	12·5	11·2	14·5	46·4	42·1	22·4	21·0	12·1	22·4
Mono- and dicarboxylic acids†	2·8	?	4·5	2·1	43·0	3·8	21·0	22·1	3·8	1·1	3·8	4·0	2·0	5·3	6·6	4·2	2·7	15·0

TABLE III*c*

	Yew	Juniper	Squash	Soybean	*Kalanchoë*	Tomato	Ivy	Avocado	Barley
Sugars									
Raffinose	—	—	2·9	—	—	—	—	—	—
Maltose	—	0·9	2·6	0·7	0·8	1·0	0·8	1·0	0·8
Sucrose	48·0	41·1	16·5	59·0	39·7	65·5	73·9	45·3	59·8
Glucose	—	7·6	0·9	0·3	—	—	—	—	—
Mannose, mannoheptulose, galactose, sedoheptulose	1·8^{SH}	2·8	0·4	—	—	—	—	15·8	—
Fructose	—	1·8	0·4	1·0	—	0·8	—	0·9	0·5
Ribulose	—	1·1	—	—	—	—	—	0·3	—
Amino-acids									
Aspartic acid	t	1·0	t	0·5	—	1·4	0·4	—	2·2
Glutamic acid	t	t	t	0·1	—	0·6	0·1	—	0·6
Serine	1·2	6·3	2·2	6·3	2·1	4·4	1·5	2·3	4·3
Glycine	?	—	0·3	—	11·1	2·0	1·7	t	2·4
Threonine	—	—	—	—	0·8	—	—	—	0·3
Alanine	27·9	5·5	10·1	3·7	1·0	11·1	9·4	1·7	14·6
Misc. below alanine	—	—	—	—	—	—	—	—	—
Miscellaneous									
Unknowns beyond alanine	—	—	—	—	—	—	—	—	—
Dextrins?	—	3·2	—	—	—	—	—	—	—
Unknown below alanine	—	—	—	—	—	—	—	—	—
Totals									
In phosphate region	9·3	15·4	69·2	17·7	37·4	4·8	10·4	26·4	10·4
Sugars	49·8	50·7	22·7	61·0	40·5	67·3	74·7	63·3	61·1
Amino-acids	29·1	17·4	12·6	10·6	15·0	19·5	13·1	4·0	24·4
Mono- and dicarboxylic acids†	6·6	13·9	3·7	8·1	3·5	6·1	0·2	3·9	3·2

t Indicates radioactive spot on film, but activity too low to count accurately.
? Indicates radioactive spot which may be compound listed but spots not distinct enough for certainty.
* 10-minute experiment.
† Activity in individual acids was not determined because spots were streaked.

environmental factors, there are variations caused by differences in the inherent metabolic rates of different organisms and differences in age of the material used, &c.

However, there are two compounds which are found to be radioactive in widely different amounts in different groups of plants. These two cases exceed by far the variations which may be attributed to the factors mentioned. One of these compounds is sucrose, the distribution of which seems to divide the plants into two distinct major groups, one in which it contains less than 10 per cent. of the soluble radioactivity and the other in which it contains more than 35 per cent. of the soluble radioactivity (Fig. 3 A, B). An inspection of the plants which constitute these two groups suggests that this difference is associated with a structural feature of the plants, namely the absence or presence of living non-photosynthetic tissue. The more rapid synthesis of sucrose in those plants containing non-photosynthetic tissues can be rationalized in terms of the selective advantage which lies in the production of a

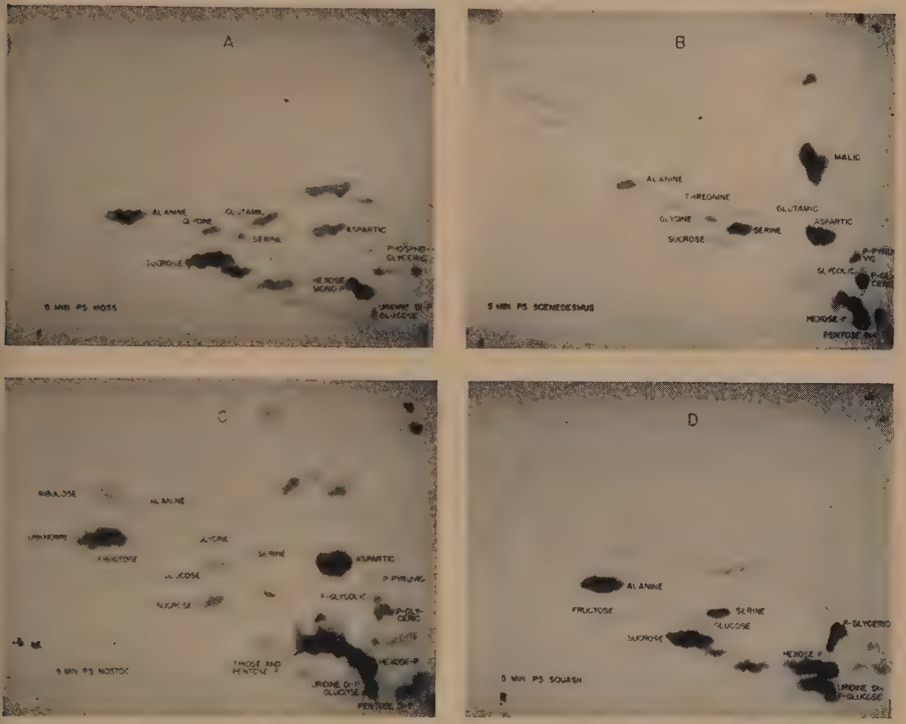

FIG. 3. Representative chromatograms from which data in Table III were obtained

A. 5 min. photosynthesis *Fontinalis* showing high concentration of sucrose.

B. 5 min. photosynthesis *Scenedesmus* showing low concentration of sucrose.

C. 5 min. photosynthesis *Nostoc muscorum* showing high concentration of unknown compound below and slightly to the left of alanine.

D. 5 min. photosynthesis squash showing absence of unknown compound below alanine.

disaccharide rather than a monosaccharide as the soluble energy source for such tissues. Because of the glycosidic link in sucrose, hexose from it may be brought into the metabolic stream without prior activation by ATP such as a monosaccharide would require. Thus the transport of sucrose carries 2-5 Kcals. more energy/mole than the equivalent carbohydrate as a monosaccharide. Evidence to support this hypothesis that the amount of disaccharide formed is related to the presence or absence of non-photosynthetic tissue in the plant rather than to its phylogenetic relationships is found in the experiments of Bean (1954) with *Iridophycus*, a multicellular red alga containing non-photosynthetic tissue. In his experiments, which were carried out in a manner comparable to that used in this laboratory, ~50 per cent. of the activity on the chromatogram was found in the disaccharide, floridoside (α-D-galacto-pyranosyl-2-glycerol).

In order to test how deep-seated the relationship between the presence or absence of non-photosynthetic tissue and the rate of sucrose production might be, experiments were done with two plants (a moss and a fern) which during one phase of their life-cycle contain no non-photosynthetic tissue but which do develop it in another phase. Presumably they might be expected to produce sucrose less rapidly during the former phase than during the latter one. In a comparison of the results of these experiments with plants in the two phases of development, it must be kept in mind that the moss protonemata and fern prothalli used were not of the same species as the mature moss and fern tested. However, the fact that the forms containing only photosynthetic tissue produced 23 and 24 per cent. sucrose (in moss protonemata and fern prothalli, respectively) and that the forms containing non-photosynthetic tissue produce 48 and 37 per cent. (in the mature moss and fern) could indicate that the potential for a higher rate of sucrose production is physiologically controlled in forms without colourless cells and does not come into full operation until non-photosynthetic tissue is developed.

The second compound which becomes radioactive in different amounts in different groups of plants is one of unknown identity which accounts for 7 and 21 per cent. of the activity of *Synechococcus* and *Nostoc* and is found (in small amounts, 1 per cent. or less) in only 9 other plants (Fig. 3 C, D). This compound runs slightly farther than alanine in the phenol direction and a somewhat shorter distance in the butanol-propionic acid direction. It is weakly ninhydrin positive and does not appear to be αε-diaminopimelic acid (Work and Dewey, 1953). Upon heating in 1 *N* H^+ at 100° C. for 1 hour at least three new substances are formed.

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Experiments on Growth in Length of Plant Organs

I. A RECORDING AUXANOMETER

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SUMMARY

An auxanometer which records automatically growth increments of 0·0009 in. is described. With the instrument changes in the rate of growth in response to changes in the gas composition of the atmosphere have been observed in periods of 10 minutes or less.

THE apparatus to be described is a modification of the auxanometer invented and used by Koningsberger (1922) in growth studies. The main component is practically identical with that of the original, but the operating mechanism, electrical circuits, and growth recorder have been simplified.

The auxanometer has been used with satisfactory results over a period of 4 years in growth experiments with coleoptiles, first by Parija (1949) investigating the effects of reduced oxygen concentrations, and later by Harrison (1953) in an extensive study of the effects of carbon dioxide.

In principle the instrument is simple. The plant is placed on the auxanometer in such a way that a very light platinum strip rests upon its tip. By its growth the plant lifts the strip to make contact with a screw in a rigid arm immediately above it. This contact completes an electrical circuit which results in the whole contact assembly being raised through a predetermined distance (0·0009 in.), breaking the circuit and resetting the instrument for the next contact when the plant has grown again through a distance equal to that moved by the contact assembly.

Each electrical contact, representing a completed growth increment of 0·0009 in., is recorded on a moving paper strip. A second marker pen inscribes a time scale of 5-minute intervals alongside the growth record, permitting the accurate determination of rapidly changing growth rates.

The apparatus is described under the headings auxanometer, electrical circuits, recorder, and accessory equipment. The latter section includes a description of a small gas chamber which was fitted to the auxanometer to facilitate the rapid replacement of the atmosphere around the growing plant.

The auxanometer. This consists of two main parts, a stationary basal portion and a moving contact assembly (Fig. 1).

The base is a heavy metal plate (A) with the main axis (B) of the instrument arising vertically from its centre to support a small adjustable platform (C),

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on which the growing plant mounted in a suitable container can be clamped. Also supported on this main axis is the electric motor (M) and associated gearing which drives the contact assembly (F, G, and J) upwards through a distance of 0·0009 in. when the contact at H is closed by the tip of the growing plant. The contacts are mounted rigidly on the top of a shaft (F) which is free to slip up or down through a cavity in the main body of the instrument. The precision with which the shaft fits into the cavity so that it may move freely in a vertical direction without the slightest angular rotation is a major determinant of the efficiency of this instrument as a whole.

Fixed to the top of the shaft (F) is a rigid horizontal arm (G) with an adjustable platinum-tipped screw (H) which forms the fixed half of the main contact. Attached to the underside of this arm, but insulated electrically from it by a paxolin plate (I), is a rolled platinum strip (J) 5 cm. long and 2 mm. wide forming the movable half of the main contact. At rest, and without the plant in position, this platinum strip rests in a horizontal position and the screw (H) is adjusted until it is about 1 mm. from the upper surface of the strip. When the plant is in position the platinum strip rests on its tip and the electrical contact is made when by its growth the plant carries up the strip to touch the screw (H). The force exerted by the platinum strip on the tip of the plant is of the order of that exerted by a 2-mg. weight in the same position.

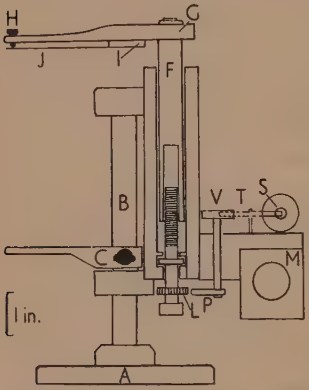


FIG. 1. A diagrammatic cross-section of the auxanometer. For explanation, see text.

The lower end of the main moving shaft (F) is drilled and tapped to take a screwed shaft fitted with a toothed wheel (L) at its lower end. This screwed shaft has a circular flange which fits exactly into a keyway cut in the main body of the instrument. On rotation of the toothed wheel (L) the screwed shaft is rotated but remains in the same position relative to the base of the instrument, while the main shaft (F) into which it is screwed moves vertically up or down carrying with it the main contact assembly through a distance which is exactly proportional to the angular rotation of the toothed wheel (L).

This toothed wheel is rotated by a pawl (P) which is driven through a gear chain by the motor (M). When the main contact is closed by the growing plant, an electrical circuit to the motor is closed. The motor operates and the pawl is driven through one complete revolution in the course of which it engages the toothed wheel (L) and drives it through the angular distance subtended by three teeth.

The toothed wheel has 100 equi-spaced teeth, and the pitch of the screwed shaft attached to it is 0·0312 in. (32 T.P.I.). The main shaft therefore is moved vertically through a distance of 0·0009 in. each time the pawl rotates when the main contact is closed.

Electrical circuits (Fig. 2). Since the growing plant organ operates directly the moving arm (J) of the main contact, it is necessary to avoid arcing at this contact when opening or closing, as this would injure not only the surface of the platinum strip but also the tip of the plant. This is obviated by the utilization of a valve-operated relay of conventional design. The main contact is in the grid circuit of a triode valve (V 1). The current flowing at any time in this circuit is extremely minute, and arcing on opening or closing is therefore cut down to a minimum. Closure of the contact causes the valve, previously biased beyond cut-off, to conduct and operate the relay (Z_1), which completes the current circuit to operate the motor (M), and the relay (Z_2) to operate the recording pen.

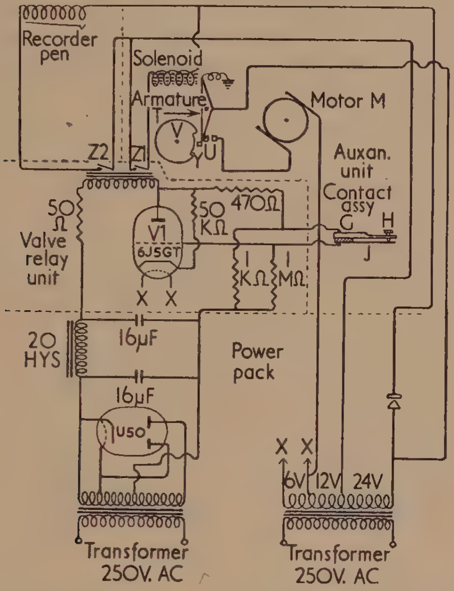


FIG. 2. A circuit diagram of the complete apparatus.

Closure of the valve relay (Z_1) activates the solenoid (S) which then attracts one end of the pivoted armature (T). At the other end, the armature (T) bears a contact (U) which closes the electrical circuit through the motor (M). The motor now operates and through a gear chain drives the pawl through one complete revolution to elevate the contact assembly. This elevation immediately opens the main contact at H and releases the dependent relays (Z_1 and Z_2), but a holding device (V) is incorporated in the motor circuit to ensure that the motor continues to operate until the pawl is returned to its starting position. The auxanometer is now reset to record the next growth measurement.

The holding device (Fig. 3) is a cam (V) mounted on the same shaft as the pawl (P). The cam-follower is attached to the armature (T) on the side opposite the motor-operating contact (U). At rest the cam-follower rests in the slot in the cam and the motor contact is open. When the main contact (H) is closed the solenoid (S) is energized via the valve relay and closes the motor contact (U), withdrawing the cam-follower from the slot. The motor operates, driving round the pawl and cam. The main contact (H) is opened almost immediately, but the cam-follower is by now riding on the raised part of the cam and the motor contact is kept closed until the cam-follower slips into the slot once again when the cam and pawl are back in the rest position.

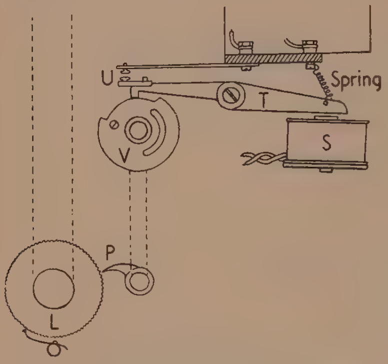
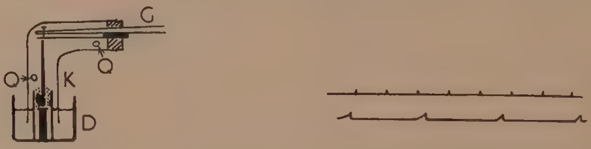


FIG. 3. Holding device in the motor circuit. For explanation see text.

The recorder. A clock mechanism is used to wind a strip of paper from one drum to another over a plane surface. Mounted over the paper are two pens. The one operated indirectly by the growing plant through contact Z_2 traces a line along the paper strip with intersections to indicate closures of the main contact (H). The second pen, operated by a timing device, traces a line parallel to the growth record with indentations at 5-minute intervals (see Fig. 4). From the record strip the growth rate can be calculated at any required time since each intercept on the growth line represents an increase of 0·0009 in. in the length of the plant.

Accessory equipment. To facilitate the rapid replacement of the atmosphere



(i) (ii)

FIG. 4. (i) A seedling mounted in the plant holder (D) and enclosed in the gas chamber (K) in position on the auxanometer. (ii) Part of a record with the upper trace showing completed growth increments and the lower trace showing 5-minute intervals.

around the growing plant, the auxanometer was fitted with a small gas chambe (Fig. 4). One arm of an L-shaped glass tube (K) was fitted over the horizonta arm of the auxanometer and held in place rigidly by a rubber bung cut t fit exactly around the horizontal arm clear of the moving contact. The vertica arm of this tube dipped down into a water-filled space between the inner an outer concentric walls of the plant holder (D). The plant mounted in th central well was thus enclosed by the glass tube in a gas space of about 25 c.c in which the atmosphere could be rapidly changed by the passage of ga streams through the inlet and outlet tubes (Q).

The auxanometer was housed in an incubator cabinet fitted with a thermo regulator and fan to maintain an even constant temperature. The power-suppl unit (see Fig. 2 for detail), valve relay, and recorder units were outside the cabinet.

Operation. Growth measurements were started by manœuvring the plant holder on the platform (C) until the tip of the plant was in the centre of the platinum strip immediately below the screw contact (H) with contact closed. With careful preparation of the plant material and adjustment of the plant holder, growth records extending over 24 hours or more were obtained without further adjustment of the instrument.

With the electrical circuit described, no passage of current could be detected through an oat coleoptile in position on the auxanometer with a galvanometer having a sensitivity of 55 cm. per microamp. at 1 metre. As a safeguard against electrical effects a platinum wire attached to the fixed end of the platinum strip resting on the plant tip was embedded in the wet sand close to the base of the plant in its holder.

Extensive comparisons of the growth rates recorded by this instrument with growth rates measured by more conventional means have led to the conclusion that the auxanometer records a true growth rate, and that coleoptile elongation is unaffected by the operation of the instrument.

Oat coleoptiles at their maximum rate of elongation in air at 25° C. recorded normally 2 to 3 increments in 5 minutes. Changes in the growth rate in response to change in the composition of the gas around the plant were usually evident after 10 minutes and often in shorter times (see Ranson and Parija, 1954).

The sensitivity of this auxanometer could be increased by a simple modification of the contact-raising mechanism. The increments recorded by Koningsberger's instrument (10 μ) were approximately half that of the instrument described, but any great increase in sensitivity beyond this point would appear to require something other than the directly operated contact mechanism used in this instrument.

Through the kindness of Professor A. F. Burstall the main body of the auxanometer was constructed in the workshops of the Department of Mechanical Engineering in King's College, Newcastle upon Tyne, by Mr. Bryson Smith, to whose interest and ingenuity the instrument owes much of its present form. The diagrams for Figs. 2 and 3 were drawn by Mr. P. Gibson of the Botany Department.

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Experiments on Growth in Length of Plant Organs

II. SOME EFFECTS OF DEPRESSED OXYGEN CONCENTRATIONS

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SUMMARY

It is shown that the growth in length of various coleoptiles, epicotyls, and hypocotyls is enhanced at oxygen tensions below that of air, though reduced when the oxygen percentage is 3·5 or less. Root extension is depressed at all oxygen percentages below 21. This effect is observed within 15 minutes.

An hypothesis to account for the differential effect at 20° C. of reduced oxygen concentration on growths of roots and shoots is outlined, and preliminary experiments to test the hypothesis are described.

Oxygen concentration had similar effects on the fresh weights of roots, coleoptiles, and epicotyls as on the lengths of these organs.

The development of lateral roots and root-hairs was retarded by reduction of the oxygen concentration to 8·5 and 3·5 per cent. In 1·5 per cent. oxygen the retardation was very marked and injury often apparent. Complete suppression of laterals and root-hairs occurred in 0·5 per cent. oxygen.

INTRODUCTION

IN the course of an investigation of the effects of reduced oxygen concentrations on the respiration of a number of plants (Ranson, 1947) it appeared that whereas the elongation of roots was less in concentrations of oxygen between 3 and 5 per cent., shoot elongation was often decidedly greater in these reduced oxygen concentrations than in air. Previous reports on the effects of oxygen on growth in general and particularly those on shoot growth were, at least superficially, conflicting and the problem invited further investigation.

Pfeffer (1900) concluded from the results of the nineteenth-century investigations that most aerobic plants fail to grow in O_2 concentrations below about 3 per cent., and that concentrations of oxygen above that in air often retard growth. He reported, however, that in oxygen concentrations between 3 and 21 per cent. growth is very commonly accelerated. In some at least of these early experiments reduced partial pressures of oxygen were obtained by a general reduction in air pressure around the plants and the results are difficult to interpret.

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More recent studies (see e.g. Cannon, 1925; Leonard and Pinckard, 1946) indicate that in general root growth is greater in air than in lower O_2 concentrations, and that below some minimum concentration in the range 0·5 to 2 per cent. growth ceases.

The effects of oxygen concentration on shoot growth are more difficult to assess, for, as Mack (1930) points out, oxygen production in light and etiolation in the dark complicate experiments with green organs. Vlamis and Davis (1943) reported, on a basis of fresh weight increase, that the growth of young rice shoots attained a maximum in 3 per cent. oxygen and that even in 0·2 per cent. oxygen growth was distinctly greater than in air. The growth of barley shoots was reported to be greater in air than in lower oxygen concentrations. However, on the basis of dry weight increase, Taylor (1942) and Lin (1946) state that growth of young shoots and roots of wheat and rice is progressively reduced in oxygen concentrations below 21 per cent., though to a less extent for the shoots than the roots.

MATERIALS AND METHODS

Growth measurements. Three main types of experiment were performed: (i) long-term experiments, in which the growth of organs attached to the seedling was measured over a period of several days; (ii) short-term experiments, in which the growth of wheat coleoptiles attached to the seedling was measured accurately and continuously with a recording auxanometer for periods of a few hours; and (iii) experiments in which the growth of detached coleoptiles was measured for periods up to 60 hours.

i. *Long-term experiments with whole attached organs.* One stock of grain or seeds of each of the following plants was used throughout the experiments: wheat ('Karn' WN 439), barley ('Plumage Archer'), rice (2 vars., 'Katacktara' and 'Rupsail'), broad bean ('Exhibition Long-pod'), vegetable marrow ('Sutton's Long White'), and buckwheat (composition unknown). About three times the required number of well-filled, and as nearly as possible equal-sized, grains or seeds were selected from the stock and immersed in 1 per cent. $HgCl_2$ solution for 20 minutes. They were then washed thoroughly in running water and allowed to swell overnight. The swollen seeds were spread out on wet cellulose wadding in a moist atmosphere to germinate. When the emerging radicles were about 1 mm. in length, replicate samples were selected. The numbers of seedlings in each sample of wheat and rice, barley and buckwheat, marrow, and broad bean were respectively 60, 40, 32, and 20.

The seedlings in a sample were mounted on a cylinder of thick blotting-paper and held, with either pins or rubber bands, in such a position that the emergent shoots and roots grew straight up and down alongside paper scales fixed to the blotting-paper cylinder. The paper cylinder was then saturated with water and placed inside a gas jar containing a 1-cm. depth of water to keep the paper saturated throughout the experiment. The jar was closed with a rubber bung fitted with inlet and outlet tubes for the circulation of gas mixtures. The seedling mounting and all subsequent examinations were performed in a dim red light. Between examinations the jars were covered with black paper.

Temperatures were controlled during growth by immersion of the gas jars in constant temperature water baths, which at 20° and 30° were thermoregulated to ±1° C. The bath at (nominally) 10° was an insulated tank containing water to which ice was added as required. The maximum fluctuation about mean temperature was ±3° C.

Growth was measured in air and in gas mixtures containing respectively 0·5, 1·5, 3·5, and 8·5 per cent. oxygen in nitrogen, passed at a rate of 5 litres per hour first through water bubblers immersed in the constant temperature baths and thence through the plant containers. Gas compositions were checked periodically with a Haldane gas analysis apparatus. There was no detectable accumulation of CO_2 in the gas jars during the experiments.

The seedling samples after transfer to the constant temperature baths were maintained in air for 12 hours and then transferred as required to other oxygen concentrations. At intervals the containers were removed from the baths for a few minutes while measurements (to the nearest 1 mm.) of root and shoot growth were made. Root measurements were confined to those first visible. In the monocotyledons these were marked at the outset to avoid confusion.

After 7 days the final lengths of the root and shoot systems were measured accurately. In experiments with wheat, rice ('Rupsail'), and broad bean at 20° C., fresh and dry weights of root and shoot systems were then determined.

ii. *Short-term experiments with whole attached coleoptiles.* Growth measurements were made over periods of a few hours with a recording auxanometer (Ranson and Harrison, 1954). Swollen wheat grains were mounted on wet cotton-wool strips in Petri dishes and incubated in darkness at 20° C. until the coleoptiles were 1·0 to 1·5 cm. in length. From this stock seeds bearing straight coleoptiles were selected in the evening and mounted, with the aid of a dim red light, in washed sterile sand in the central well of the auxanometer plant holder with the coleoptile pointing vertically upwards. The sand was saturated with water and the outer well of the plant holder filled with water. Several coleoptiles mounted in this way were conveyed in darkness to the incubator containing the auxanometer and allowed to equilibrate overnight. Any coleoptiles which developed visible curvatures in this period were discarded. The remainder were used as required for growth measurements.

The plant holder was placed on the auxanometer with the tip of the coleoptile impinging on the tip of the platinum contact and the lower lip of the gas holder immersed in the water in the outer well of the plant holder. The auxanometer contact assembly was screwed down until the first contact was made. Thereafter the instrument operated automatically recording the coleoptile growth increments (each growth increment = 0·0009 in. or 23 μ) for periods of 12 to 24 hours, often without further adjustment of the coleoptile.

Gas streams were passed from cylinders at a controlled rate of about 5 litres per hour, first through a long column of water inside the auxanometer incubator, and then through the small gas chamber around the coleoptile.

Growth rates were recorded in air for several hours before and after growth periods in other gas mixtures. The small volume of the gas chamber (about 25 ml.) allowed virtually complete replacement of the atmosphere about the coleoptile within 5 minutes. Changes in growth rate in response to the change of atmosphere were often evident within 15 minutes.

iii. *Experiments with detached coleoptiles.* Wheat grains were germinated and grown in air as previously described until the coleoptiles were 1·5 to 2·0 cm. long. The coleoptiles were then detached, the enclosed leaves removed, and the coleoptiles mounted in samples of 10 on the teeth of glass combs (see Schneider, 1938). Each comb with its coleoptile complement was placed in a Petri dish containing 40 ml. of the appropriate bathing solution. The coleoptiles were immersed partially along their whole length in the bathing fluid. The Petri dishes were covered with transparent perspex lids pierced by gas inlet and outlet tubes for the circulation of the different gas mixtures. The lids were sealed to the Petri dishes with wax and the gas inlet tubes adjusted to dip below the surface of the fluid in the dish. Growth of the coleoptiles was measured at 6-hour intervals using a travelling microscope fitted with a micrometer eyepiece. The Petri dishes were maintained at constant temperatures and, except during the mounting and subsequent measurements carried out in dim red light, in darkness.

Determination of ethyl alcohol accumulations. The seedlings after periods of growth in the required oxygen concentrations were steam distilled, and the volatile products in the distillate oxidized with sulphuric acid-bichromate solution after the method of Thomas (1925). Alcohol was measured as acetic acid by titration. The alcohol contents so determined may include a small component attributable to steam volatile substances (e.g. acetaldehyde) other than ethyl alcohol.

Preliminary attempts at auxin assay. Wheat grains were germinated in air and then transferred to the appropriate gas mixture to continue growth. On the day of maximum growth for the particular oxygen concentration in use (see Fig. 1) 1,200 coleoptiles were cut from the seedlings, freed from enclosed plumules, and macerated in a blender with 250 ml. of peroxide-free diethyl ether. The macerate was stored overnight at about 0° C. The ether was decanted and the residue extracted again at room temperature with two successive 100-ml. portions of ether for 2-hour periods. The ether extracts were combined, the ether removed at reduced pressure, and the residual lipoidal mass extracted with water. The aqueous extract was made up to 40 ml., 400 mg. of sucrose added, and the solution poured into a Petri dish. The auxin content was assayed by the straight-growth technique (Bonner, 1933; Schneider, 1938), each Petri dish containing for this purpose 40 × 3 mm. sections of wheat coleoptile previously grown under the standardized conditions in air. During the assay a stream of air was passed through the Petri dish and bathing solution. Because of the labour involved extracts from coleoptiles grown in different oxygen concentrations were prepared on successive days and stored at 0° C. until they could be assayed simultaneously.

Expression of results. In the following sections for the long-term experiments maximum daily rate means the growth increment recorded on the day when growth was fastest and final length means the length of the plant organ at the end of an experiment, i.e. usually after 7 days' growth in a particular oxygen concentration. Table I shows typical results derived from single experiments with each plant type, and rates and lengths given are means for

TABLE I

Maximum daily rates of growth (cm. per day) and the final lengths (cm.) of plant organs grown in air and in 8·5, 3·5, 1·5, and 0·5 per cent. oxygen at 10°, 20°, and 30° C.

Plant organ	Temp.	Max. daily rate					Final length				
		21%	8·5%	3·5%	1·5%	0·5%	21%	8·5%	3·5%	1·5%	0·5%
Wheat root	10°	0·31	0·22	0·24	0·17	0·10	0·79	0·56	0·46	0·44	0·15
	20°	1·42	1·03	0·90	0·80	0·14	7·01	4·09	3·62	2·31	0·37
	30°	0·98	0·88	0·68	0·37	0·26	5·13	4·28	3·24	1·37	0·60
Barley root	10°	0·27	0·20	0·12	0·15	0·11	0·60	0·36	0·23	0·26	0·17
	20°	1·32	1·17	1·04	0·52	0·28	6·62	4·90	4·38	2·32	0·94
	30°	0·81	0·75	0·73	0·33	0·13	3·48	3·00	2·46	1·20	0·45
Rice ('Katacktara') root	10°	0·51	0·36	0·37	0·50	0·22	0·98	0·77	0·77	0·95	0·50
	20°	1·67	1·51	1·25	0·83	0·31	8·87	7·34	6·05	3·85	1·26
	30°	1·78	1·62	1·52	0·92	0·37	9·84	8·53	7·73	4·53	1·45
Rice ('Rupsail') root	10°	0·41	0·45	0·37	0·20	0·12	1·96	1·10	0·81	0·50	0·32
	20°	1·75	1·36	1·05	0·75	0·35	6·32	5·00	3·86	2·95	1·21
	30°	1·90	1·52	1·37	1·05	0·31	7·15	5·77	4·96	3·90	1·22
Marrow root	10°	0·25	0·22	0·20	0·18	0·15	0·55	0·54	0·43	0·38	0·23
	20°	1·37	1·17	0·99	0·59	0·23	7·10	5·87	5·28	2·76	0·83
	30°	1·18	1·15	1·85	0·63	0·31	6·50	5·78	4·87	2·81	1·00
Buckwheat root	10°	0·20	0·20	0·15	0·16	0·15	0·43	0·34	0·27	0·21	0·28
	20°	1·51	1·20	0·93	0·56	0·34	7·43	5·80	4·80	2·72	1·12
	30°	1·22	0·88	0·65	0·59	0·46	6·21	4·60	3·72	2·77	1·29
Broad bean root	10°	0·32	0·34	0·15	0·11	0·10	0·93	0·97	0·35	0·30	0·20
	20°	1·68	1·34	0·94	0·50	0·15	6·38	4·89	3·95	2·37	0·45
	30°	1·40	1·15	0·75	0·42	0·36	5·9	4·75	3·64	2·34	0·78
Wheat coleoptile	10°	0·30	0·27	0·25	0·15	0·10	0·68	0·59	0·54	0·32	0·20
	20°	1·42	1·78	2·02	0·78	0·22	4·71	7·02	8·55	2·32	0·64
	30°	1·24	1·50	1·72	0·65	0·25	4·16	5·57	7·25	2·56	0·84
Barley coleoptile	10°	0·26	0·20	0·20	0·10	0·11	0·61	0·50	0·52	0·27	0·26
	20°	1·46	1·69	1·41	0·64	0·25	5·06	6·77	6·23	2·52	0·91
	30°	1·35	1·60	1·41	0·61	0·38	4·90	5·91	6·32	2·46	1·27
Rice ('Katacktara') coleoptile	10°	0·20	0·21	0·10	0·13	0·11	0·50	0·46	0·27	0·25	0·18
	20°	0·68	0·89	0·90	0·51	0·31	2·33	3·21	4·02	2·35	0·95
	30°	0·85	0·95	0·98	0·61	0·45	3·22	3·78	4·87	2·87	1·56
Rice ('Rupsail') coleoptile	10°	0·11	0·10	0·12	0·10	0·05	0·21	0·25	0·20	0·18	0·10
	20°	0·68	0·78	0·92	0·64	0·38	1·72	2·80	3·78	2·96	1·55
	30°	0·80	1·85	1·12	0·81	0·45	2·33	3·21	4·24	3·49	1·81
Marrow hypocotyl	10°	0·25	0·20	0·15	0·10	0·10	0·50	0·41	0·33	0·22	0·18
	20°	1·18	1·75	1·60	0·67	0·21	4·39	6·30	5·71	1·58	0·50
	30°	0·90	1·32	1·15	0·37	0·32	4·28	4·97	4·90	1·11	0·74
Buckwheat hypocotyl	10°	0·26	0·25	0·17	0·15	0·10	0·75	0·60	0·45	0·35	0·23
	20°	1·29	2·20	2·15	0·88	0·25	4·17	10·06	9·55	3·71	0·66
	30°	0·85	0·81	0·78	0·45	0·26	2·59	4·11	3·35	2·23	0·56
Broad bean epicotyl	10°	0·15	0·12	0·15	0·08	0·05	0·30	0·25	0·33	0·16	0·10
	20°	0·70	1·10	0·82	0·36	0·10	2·08	2·70	2·37	1·21	0·28
	30°	0·56	0·75	0·60	0·40	0·12	1·78	2·16	2·30	1·27	0·22

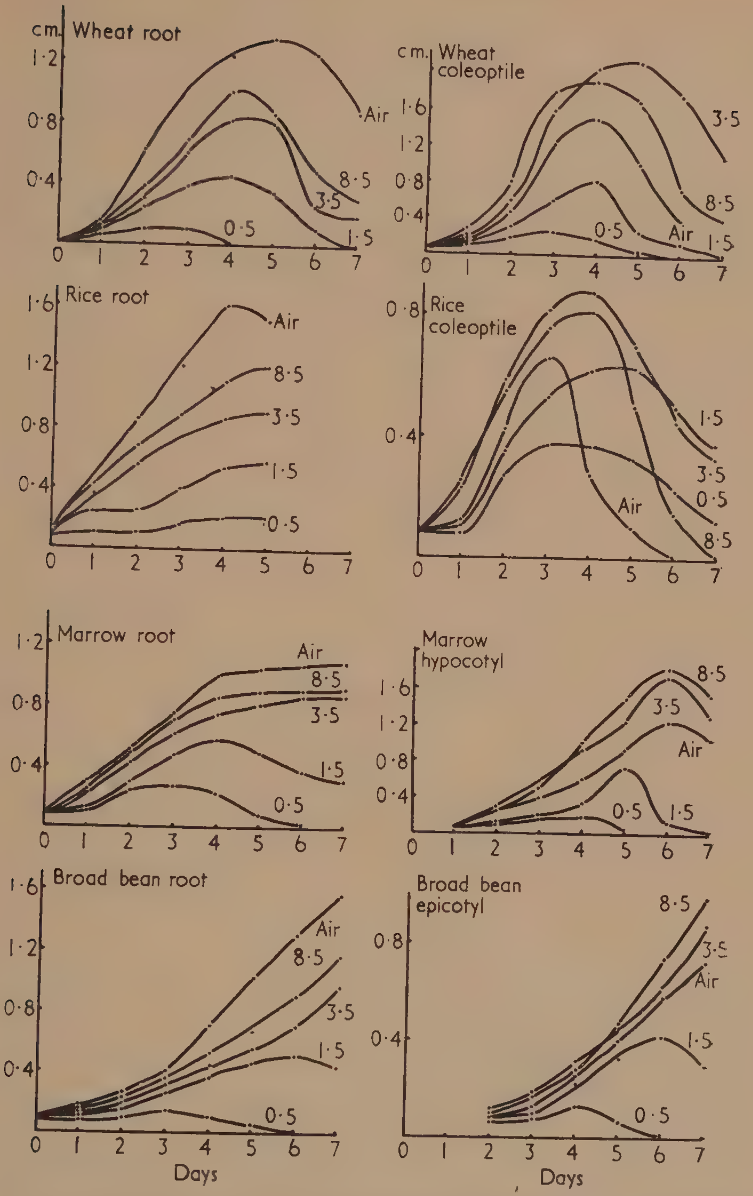

FIG. 1. Growth rates in cm. per day of roots and shoots of wheat, rice ('Rupsail'), marrow, and broad bean, in air and in 8·5, 3·5, 1·5, and 0·5 per cent. oxygen at 20° C.

the seedlings in samples subjected to each gas. Fig. 1 is a partial record of another set of experiments at 20° C.

For the short-term experiments the auxanometer records have been analysed and the results are presented in the charts given in Fig. 2 as the numbers of 23 μ growth increments completed in successive 15-minute intervals.

EXPERIMENTAL RESULTS

The effect of oxygen concentration on growth in length of roots. At 20° C. (see Table I) the maximum daily rates of growth and the final lengths of all roots studied decreased with each decrease in the O_2 concentration below that of air. In 0·5 per cent. O_2 (see Fig. 1) root growth ceased within 6 days except in the 'Rupsail' variety of rice. In all root systems and particularly in those of the dicotyledons the number of root-hairs and lateral roots was reduced with each decrease in oxygen concentration below 21 per cent. The inhibition was marked at 1·5 per cent. O_2 and, except on roots of rice ('Rupsail'), root-hairs were not visible in 0·5 per cent. O_2. In 3·5 per cent. O_2 and above root systems appeared to be healthy, but below this concentration signs of injury were often visible; wheat roots turned brown at the tips, roots of vegetable marrow developed nodular structures, and broad bean roots sometimes curled into knots at their tips. At 30° C. the effects of O_2 concentration were broadly similar to those at 20°. At 10° C. there was little or no growth in any oxygen concentration after 3 days. The roots of the rice varieties were the only ones of those investigated in which the lengths after 7 days were greater in all oxygen concentrations at 30° than 20° C.

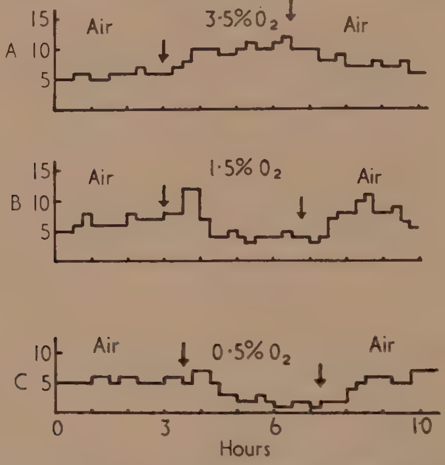

FIG. 2. Growth rates at 20° of wheat coleoptiles in air, 3·5 (A), 1·5 (B), and 0·5 per cent. oxygen (C), as recorded by the auxanometer. Growth rates (abscissae) are recorded as the numbers of growth increments (each 23 μ) completed in 15-minute intervals. Times of transfer from one oxygen concentration to another are indicated on the time scale (ordinates) by vertical arrows.

The effect of oxygen concentration on growth in length of shoot organs. (i) *Long-term experiments.* At 20° C. the maximum daily growth rates and final lengths of the coleoptiles, hypocotyls, and epicotyls were markedly increased by reduction in oxygen concentration below 21 per cent. Maximum rates and final lengths occurred in all cases in either 8·5 or 3·5 per cent. O_2. Except for the rice coleoptiles growth was less in 1·5 per cent. O_2 than in air, and markedly retarded in 0·5 per cent. oxygen. Broadly, the effects of reduced oxygen concentrations were similar at 30° to those at 20° C., but at 10° no stimulation of growth was detectable in any O_2 concentration below 21 per cent.

(ii) *Short-term experiments.* The effects of oxygen concentration on the elongation of wheat coleoptiles at 20° C. are illustrated by the records of three auxanometer experiments in Fig. 2. In air growth rates fluctuated consistently about a mean value. The amplitude of the fluctuation was rarely more than 10 per cent. of the mean rate and no very regular rhythm could be detected. Similar variations in rate have been observed in auxanometer records of a large number of experiments with oat coleoptiles (Harrison, 1953). The fluctuations do not appear to be related to any changing environmental condition and they may be an inherent characteristic of coleoptile extension.

On replacement of the air atmosphere around the coleoptile with 3·5 or 8·5 per cent. O_2 the growth rate increased within 30 minutes. After about 1 hour the rate levelled off at a value very often more than double the previous rate in air. On return to air it decreased gradually during 2 or 3 hours to a rate near the original rate in air. FIG. 2A records a typical result.

In 1·5 per cent. oxygen (FIG. 2B) there was often, though not invariably, within the first hour after transfer a short period of more rapid growth. Invariably after 2 or 3 hours it was about half that recorded in air. On replacement in air the rate returned within a further 2 or 3 hours to a value near the original rate in air. In 0·5 per cent. O_2 the growth rate decreased, again sometimes after a temporarily increased rate, to a value about one-fifth of the rate in air. Return to air after a few hours at this low level caused an immediate increase in growth rate. In pure nitrogen growth rates declined rapidly, and usually within about 1 hour growth apparently ceased (i.e. no growth increment was recorded within 3 hours). On return to air after 3 to 6 hours growth recommenced immediately.

Often, on return to air from oxygen concentrations of 1·5 per cent. and below, growth rates increased rapidly to rates well above normal air values for a short period and then decreased again towards the normal value. Whether or not the temporarily increased rates of growth on transfer from air to low oxygen concentration, or on transfer from the low oxygen concentration back into air, are a result of a gradual change of the oxygen tension within the coleoptile or are in fact transient effects of the substituted oxygen concentration is as yet unknown.

The effects of oxygen concentration on fresh weights of seedling parts at 20° C. The fresh weights of complete root systems of wheat, rice ('Rupsail'), and broad bean after 7 days' growth decreased with each decrease in oxygen concentration from 21 to 1·5 per cent. (see Table II). The fresh weights of wheat and rice ('Rupsail') coleoptiles and of broad bean epicotyls were increased with decrease in the oxygen concentration from 21 to 3·5 per cent., but in 1·5 per cent. O_2 only the rice coleoptile fresh weight was greater than that in air. The effect of oxygen concentration on these seedling organs was thus broadly similar to the effect on the final lengths. No regular drift with oxygen concentration was, however, discernible in the fresh weight/final length quotients.

Unlike those of the coleoptiles, the fresh weights of the leaves enclosed within the coleoptiles were reduced in oxygen concentrations lower than that of air. Vlamis and Davis (1943) reported for rice shoots fresh weights of the order of 170 per cent. of those in air after growth in 2·7 to 5·2 per cent. O_2.

TABLE II

Mean fresh weights in mg. of the component parts of seedlings of wheat, rice ('Rupsail'), and broad bean, after 7 days' growth in darkness at 20° C. in air, 8·5, 3·5, and 1·5 per cent. oxygen

		Fresh weights			
Plant	Part	21%	8·5%	3·5%	1·5%
Wheat	root	455	405	325	180
	coleoptile	310	485	560	170
	leaf	800	730	650	135
	total shoot	1,110	1,215	1,210	305
	root+shoot	1,565	1,620	1,535	485
Rice ('Rupsail')	root	88	75	69	48
	coleoptile	99	125	145	138
	leaf	157	135	118	95
	total shoot	256	260	263	233
	root+shoot	344	335	332	281
Broad bean	root	3,970	3,150	2,300	1,990
	shoot	5,880	6,520	6,950	3,140
	root+shoot	9,850	9,670	9,250	5,130

The relationship between fresh weight of shoot and oxygen concentration is, however, the resultant of apparently dissimilar trends with oxygen concentration in the fresh weights of the coleoptiles and the enclosed leaves. Hence the relative weights of shoot systems grown in different oxygen concentrations may vary with the stages of development of the coleoptile and plumule. Fresh weights of root and shoot together (see Table II) were not very different after 7 days' growth in 21, 8·5, or 3·5 per cent. oxygen.

Determination of the extinction points of anaerobic respiration. The extinction points of anaerobic respiration of the marrow and buckwheat seedlings are at values about 2 per cent. oxygen (Ranson, 1947). As qualitative differences in metabolism might account for the differences in growth behaviours above and below this oxygen concentration, extinction points were determined for the remainder of the seedlings. Ethyl alcohol contents of seedlings grown for 48, 96, and 144 hours in a variety of oxygen concentrations were determined (Table III) and the accumulation of alcohol taken as a measure of anaerobic metabolism in a particular concentration (Thomas and Fidler, 1933).

There were appreciable anaerobic components in the respiration of wheat, rice ('Katacktara'), and broad bean in 1·5 but not in 3·5 per cent. oxygen, indicating that the extinction points were between these values. In rice ('Rupsail') seedlings alcohol values in 1·5 per cent. O_2 after 48 hours were distinctly greater than those in higher oxygen concentrations, but in subsequent periods there was no further alcohol accumulation. In 0·5 per cent. O_2 there was positive and continued accumulation throughout the 144 hours' exposure, consequently the extinction point appears to be close to 1·5 per cent. oxygen and thus at a somewhat lower value than for other seedlings. The inference is that for each of the plants studied such changes in growth response as have been noted previously in oxygen concentrations below 3·5 per cent. occurred when the oxygen level was such that anaerobic metabolism became appreciable.

TABLE III

Ethyl alcohol contents (in mg.) of seedlings, derived from 25 g. air dry weight of seed, measured after 48, 96, and 144 hours' growth in gas mixtures containing 21, 8·5, 3·5, 1·5, and 0·5 per cent. oxygen, and in pure nitrogen

Plant	Exposure time (hrs.)	Oxygen concentration					
		21%	8·5%	3·5%	1·5%	0·5%	0·0%
Wheat	48	8	8	10	17	63	—
	96	8	10	12	39	151	—
	144	7	10	14	59	368	—
Rice ('Katacktara')	48	3	3	4	16	56	—
	96	3	3	5	35	88	—
	144	3	7	11	50	156	—
Rice ('Rupsail')	48	2	2	3	14	13	150
	96	2	3	4	15	47	170
	144	4	7	9	17	53	(72 hrs.)
Broad bean	48	5	6	8	31	113	—
	96	8	13	13	40	287	—
	144	8	13	19	87	313	—

The effects of oxygen concentration on the elongation of wheat coleoptiles detached from the seedling. The different effects of 8·5 and 3·5 per cent. oxygen concentrations on the elongation of root and shoot organs appeared to rule out any effect of oxygen concentration on respiratory activity as a major cause of the results described. Among the accepted ideas at the time of these experiments (see e.g. Went and Thimann, 1937) were that (*a*) the growth of plant organs is related to the concentration of free auxin in the growing zone, (*b*) within certain limits increase in auxin leads to increased growth in shoot systems but, above some low concentration, to decreased growth of roots, and (*c*) 'one of the most marked chemical properties of the auxins is their extreme sensitivity to oxidative destruction'. On the basis of these ideas Professor Thomas suggested that the observed effects of oxygen on growth might be accounted for if reduction in oxygen leads to a decreased oxidative consumption of auxin. If then the auxin controlling growth is the balance of auxin produced over auxin consumed, reduced oxidative consumption would leave more available for growth, so, possibly, producing increased shoot growth and decreased root growth.

In experiments to test this theory wheat coleoptiles were used, and as a preliminary the effects of oxygen concentration on the elongation of whole coleoptiles detached from the seedling were investigated. Fig 3A shows the means of the percentage increases in length of four samples of whole detached wheat coleoptiles (initially about 2 cm. in length) grown at 20° C. in 1 per cent. sucrose solutions through which gases containing the different oxygen concentrations were passed. The results shown are typical of those obtained in three such experiments. The rates of growth in the first 18 to 24 hours were distinctly faster in both 3·5 and 8·5 per cent. O_2 than in air. Growth in 1·5 per cent. O_2 was always slower than in air. The effect of oxygen concentration on the growth of detached coleoptiles was thus in the main similar to that on coleoptiles attached to the seedling. A possible explanation of these results would be a greater oxidative consumption of residual auxin in the detached coleoptiles in the higher oxygen concentrations.

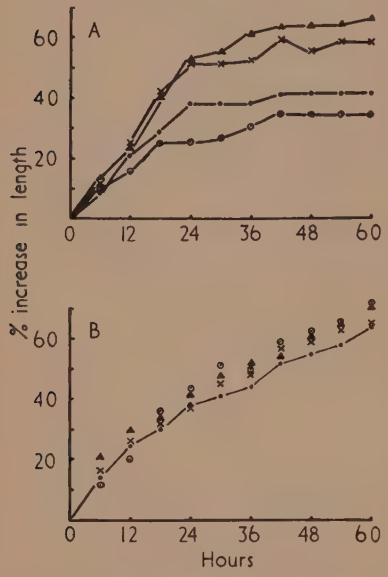

Fig. 3. Mean percentage increases in length with time in hours of wheat coleoptiles immersed in (A) 1 per cent. sucrose solution, and (B) 1 per cent. sucrose solution containing 1 mg./litre IAA, in air (●—●), 8·5 (×—×), 3·5 (▲—▲), and 1·5 (⊙—⊙) per cent. oxygen. Temperature 20° C.

In another series of experiments whole detached coleoptiles were grown in four oxygen concentrations in a sucrose solution containing 1 mg. β-indolyl acetic acid (IAA) per litre. It was argued that if the growth rates in different oxygen concentrations resulted from different rates of oxidative consumption of natural auxin, then the provision of an abundant external supply of growth substance (IAA) might conceivably nullify the oxygen effect. The results of one experiment of this type are illustrated in Fig. 3B. In each gas mixture the coleoptiles extended with little diminution in rate throughout the 60 hours of the experiment, but the rate of extension was not consistently higher in any one oxygen concentration. Consequently, it is concluded that in the presence of an abundant external supply of IAA, growth rates of detached coleoptiles were not greatly affected by oxygen concentration in the range 1·5 to 21 per cent.

Attempted assay of auxin in wheat coleoptiles growing in different oxygen concentrations. In an attempt to obtain a measure of the auxin present in coleoptiles in different oxygen concentrations assays were made by simple techniques of the auxin contents of samples of coleoptiles cut from seedlings growing in different oxygen concentrations at the times of their maximum growth rate. Table IV shows the mean percentage elongations of samples of forty coleoptile sections grown in 1 per cent. sucrose alone, with addition of 1 mg. per litre

IAA, and with addition of extracts of coleoptiles grown in air, 8·5, 3·5, and 1·5 per cent. oxygen. In each of the solutions containing extracts from coleoptiles grown in air, 8·5 and 3·5 per cent. O_2 there was some evidence of growth promoting power. There was no evidence of growth-promoting power in the extract from coleoptiles grown in 1·5 per cent. O_2. Auxin may have been absent or, if present, its action suppressed by an inhibitor. Clearly in these results there is no evidence of presence of more growth promoting power in coleoptiles growing at a more rapid rate in 8·5 per cent. O_2 than in those growing in air. The extract from coleoptiles grown in 3·5 per cent. oxygen may have contained more growth-promoting substance than the air extract; but, since the hypothesis formulated requires that more growth promotion be found in extracts of coleoptiles from both 3·5 and 8·5 per cent. oxygen than from those grown in air, the results are indecisive.

TABLE IV

Mean percentage elongations with time of coleoptile sections aerated in 1 per cent. sucrose solution alone (S) and with additions of 1 mg./litre IAA (S+IAA), and extracts from coleoptiles grown in air (E_{air}), 8·5 ($E_{8·5}$), 3·5 ($E_{3·5}$), and 1·5 ($E_{1·5}$) per cent. oxygen

Time in hours	Bathing solutions					
	S	S+IAA	E_{air}	$E_{8·5}$	$E_{3·5}$	$E_{1·5}$
6	10	15	10	14	11	11
12	18	24	15	20	22	15
18	22	41	27	20	30	22
24	25	52	35	30	30	30
30	34	52	38	40	35	30
36	34	54	42	42	44	30
42	34	59	44	42	48	30
48	34	64	44	42	48	30
54	34	65	44	42	50	30
60	34	67	44	42	50	30

DISCUSSION

It has been shown that at 20° C. reduction of the oxygen concentration in the atmosphere around young seedlings had quite different effects on the growth of shoot organs and roots. In all plants studied reduction in oxygen concentration below that in air caused increased growth in length of coleoptiles, hypocotyls, and epicotyls until the extinction point of anaerobic respiration was reached at some oxygen concentration below 3·5 per cent. Below the extinction point growth was retarded progressively with increase in the anaerobic component of the total metabolism. In root systems any decrease in oxygen concentration below that in air caused a decreased growth in length.

Experiments with the auxanometer confirmed the results for wheat coleoptiles and showed that the growth rate changed in the course of a few minutes in response to change in oxygen concentration. Within 1 hour of the transfer from air the rate of growth in 3·5 and 8·5 per cent. oxygen was often double that in air before transfer. Transitory changes in growth rates not apparent in the long-term experiments were often evident in the auxanometer records. If, as suggested earlier, the changes in growth rate result from changes in the auxin available for growth then, presumably, transitory fluctuations in growth rate reflect similar changes in auxin concentration.

Fresh weights of shoot organs and roots were influenced in the same ways as the lengths of the organs by the various oxygen concentrations, but the fresh weights of shoot and root together were very little different for wheat, rice ('Rupsail'), and broad bean seedlings grown in air, 8·5, or 3·5 per cent. oxygen.

To explain the difference in growth behaviour of root and shoot organs with reduction of oxygen concentration in the range 21 to 3·5 per cent. it has been suggested that reduction in oxygen concentration, by leading to a reduction in the oxidative destruction of auxin, might increase the effective auxin concentration in growing zones, so increasing shoot growth and decreasing root growth. Experiments with detached coleoptiles provided a measure of support for the hypothesis in that in the presence of abundant growth-promoting substance (IAA), coleoptile growth was not markedly affected by the oxygen concentration. The two auxin assays attempted, however, provided no evidence of real value of the presence of more growth-promoting power in extracts from coleoptiles growing in 3·5 and 8·5 per cent. oxygen than in those growing in air. Moreover, the retardation of growth (as indicated by fresh weight measurements) of the plumule leaves within wheat and rice coleoptiles in oxygen concentrations below 21 per cent. in which the coleoptile growth is accelerated, is a differential effect of oxygen concentration on the growth of shoot organs for which the hypothesis supplies no explanation.

Since this work was carried out new and much improved methods, e.g. the chromatographic technique of Bennet-Clark and Kefford (1953), have been developed for the analysis of natural auxins in crude extracts. With such techniques ether extracts of several plants have been shown to contain complex mixtures of growth-promoting and growth-inhibiting substances. It is perhaps not surprising therefore that no well-defined relationship between growth rates in various oxygen concentrations and extracted growth-promoting power was disclosed in our simple assay experiments. Obviously the hypothesis that the effects of oxygen concentration on the growth of root and shoot systems are a result of changes in rates of oxidative consumption of auxin is only one of the explanations which might be formulated in terms of growth promoters and growth inhibitors.

The authors' thanks are due to Professor M. Thomas for many suggestions and discussions throughout this investigation.

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Changes in the Resistance of the Mesophyll Tissue with Changes in the Leaf Water Content

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SUMMARY

Porometer measurements of the resistance offered to viscous flow of air by mesophyll tissue rings cut from leaves were carried out simultaneously with determinations of changes in tissue water content. Decreases in water content were accompanied by increases in mesophyll resistance and these changes are discussed in relation to porometer measurements of total resistance offered by leaves to viscous flow of air.

INTRODUCTION

DURING porometer measurements of total resistance offered by leaves to viscous flow of air, attempts were made to assess the resistance of the mesophyll tissue of hypostomatous leaves which underwent changes in their water content. The resistance of the mesophyll tissue was measured by placing an annulus of tissue cut from fully turgid leaves between gelatine washers of a porometer clamp. Results indicated that measurable changes in the resistance of the mesophyll tissue occurred and it was decided to investigate these under controlled conditions in the laboratory.

APPARATUS

The porometer which was used for the investigations was based on the mechanism of Maskell's air-flow meter. The 1·0-mm. diameter capillary tube *A* was fused to a three-way stopcock *B* and at its end *C* the bore was greatly reduced. This constriction served both as an air-escape vent and as a trap for the 15·0-mm. long column of mercury *M*, preventing its escape from the tube. On account of the small orifice at *C* the descent of the mercury column over 50·0 cm. of the tube lasted 3·5 seconds and a maximum of 0·392 cm.^{3} of air was drawn into the apparatus. The mercury was raised to its starting position below the stopcock of the instrument by applying a rubber medicine dropper bulb at *C*.

The attachment clamp *D* consisted of a suitably tempered steel clip, 3·0 cm. wide, the tension of which could be regulated by screw *E*. The pressure of the clamp was about 200 g. per cm.^{2} A piece of perspex was fixed to the lower jaw of the clip and this fitted over the rubber-covered end *H* of tube *A*. The centre hole *F* of 3·0 mm. radius was concentric with a groove *G* ($r' = 4·0$ mm., $r'' = 6·0$ mm.). The upper jaw of the clip was fitted with a corresponding piece of perspex without the centre hole.

An expression of the resistance ρ, between a pair of opposite edges of

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mesophyll tissue squares, 1·0 cm. × 1·0 cm. in extent, could be calculated from the resistance offered by *narrow* tissue rings with the aid of the formula:

$$\rho = \frac{2\pi R}{\log_e r'/r''},$$

where R is the resistance of an annulus to radial flow and r', r'' are the radii of the annulus. For variable annuli of tissue this calculation becomes more complicated and involves Bessel functions (Penman, 1942).

The calibration of the instrument was carried out by attaching a series of capillary resistances of known Gregory and Pearse unit values to the attachment clamp. These resistances were made from cylindrical thermometer tubing the lengths of which were calculated by means of the formula quoted by Heath (1939). The conductance of these thermometer tubes was in addition measured by Barr's (1934) method. A water manometer was connected to the side tube N, below the stopcock, in order to ascertain the relation between the time of descent of the mercury, the pressure drop from the outside to the inside of the instrument, and the resistance in Gregory and Pearse units. The descent of the mercury over 50·0 cm. was timed to the nearest half-second. An increase of 1·0 Gregory and Pearse unit resistance corresponded to an increase of 5·5 seconds in the time of descent of the mercury. Therefore differences of 0·1 Gregory and Pearse units could be measured satisfactorily. The instrument reached the limit of its accuracy when the time of descent reached about 275 seconds for 50·0 cm., corresponding to about 50 Gregory and Pearse units.

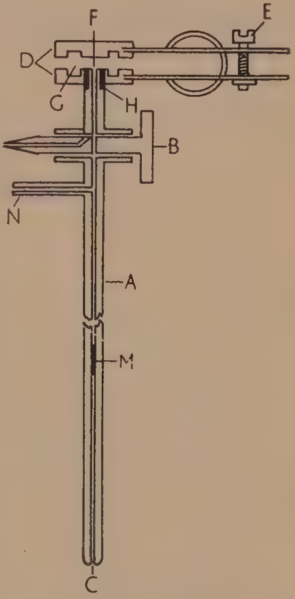

FIG. 1. Longitudinal section of mercury drop porometer.

A. Capillary tube. B. 3-way stopcock. C. Air escape vent. D. Attachment clamp. E. Pressure regulating screw. F. Central hole. G. Grooves for washers. H. Rubber joint. M. Mercury column. N. Manometer attachment tube.

Experimental methods. Twigs were prepared by cutting them under water and stripping their cut ends of bark (Haines, 1936). These twigs were kept overnight with their cut ends in water and on the following day tissue rings ($r' = 4{\cdot}0$ mm., $r'' = 6{\cdot}0$ mm.) were cut out of leaves with razor-sharp punches. Inclusions of large veins in these rings were avoided. The rings were weighed on an aperiodic balance and this initial weight was considered to be the weight of the rings when fully turgid. The tissue rings were next transferred to a chamber filled with an atmosphere saturated with water vapour, where they were fitted between gelatine washers of the porometer clamp. The gelatine washers (Gregory, 1950) were prepared without glycerine since the danger of their drying out was excluded when working in an atmosphere saturated with water vapour.

The resistance to viscous flow of air offered by tissue rings was determined by timing the descent of the mercury over 50·0 cm. Constant readings were obtained within 30 seconds after fitting the rings into the attachment clamp. The rings were next placed on the aperiodic balance for the second time and allowed to lose a certain percentage of their weight before being returned into the attachment clamp of the porometer for a second set of measurements. This procedure was repeated four or five times until the total loss in weight of the rings was estimated to have reached about 10 per cent. of the amount of water contained in the fully turgid rings. Finally the rings were dried at 105° C. in order to establish their dry weight, and by calculation the amount of water contained in a fully turgid ring.

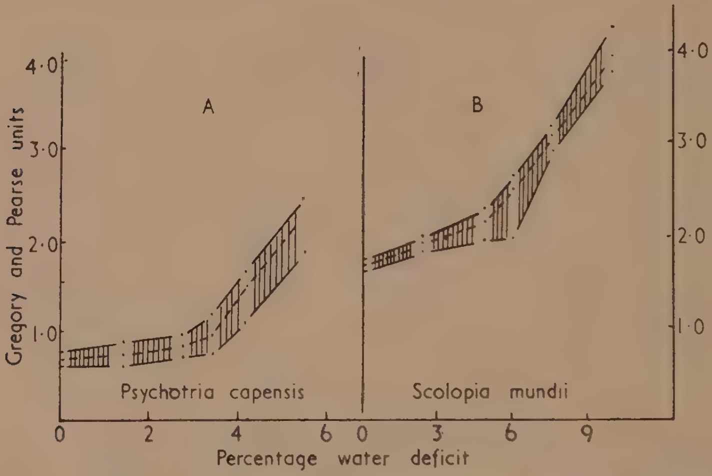

FIG. 2. Changes in the resistance of mesophyll tissue rings with changes in their water contents.

Thus all water deficits mentioned in this paper are percentages of the total water contained in fully turgid tissues.

Of each leaf species twelve leaves were used, and from each of these four tissue rings could be cut without including major veins. Measurements of the resistance offered to viscous flow of air were carried out at five different water content levels with each ring. A total of 240 readings was thus obtained for each leaf species.

RESULTS

The 240 readings plotted in Fig. 2, A and B, are all situated in the shaded areas; the dotted lines connect the calculated mean values for different water content levels.

Psychotria capensis. The average thickness of the hypostomatous leaves of this 'dense shade' plant was 290 μ. Mesophyll tissue rings ($r' = 4{\cdot}0$ mm., $r'' = 6{\cdot}0$ mm.) when fully turgid offered a resistance to viscous flow of air of 0·6 Gregory and Pearse units. A tissue with such a low resistance was found well suited for an investigation into gradual increases in this resistance with decreases in water content.

The rate of increase of the resistance of the mesophyll tissue to viscous flow of air changed rather markedly after the amount of water contained in a fully turgid ring had decreased by 3·5 per cent. Leaf water deficits of up to 4·0 per cent. were commonly met with during the course of investigations with *Psychotria capensis* in the field.

Scolopia mundii. The hypostomatous leaves of this 'open shade' plant had an average thickness of 220 μ. The resistance of mesophyll tissue rings ($r' = 4{\cdot}0$ mm., $r'' = 6{\cdot}0$ mm.) when fully turgid was 1·6 Gregory and Pearse units. The rate of increase of this resistance changed when the amount of water contained in a fully turgid ring had decreased by 5·1 per cent. Leaf water deficits of up to 6·2 per cent. occurred commonly in the field.

DISCUSSION

Results shown in Fig. 2 indicate that increases in the resistance of the mesophyll tissue as measured with a porometer occurred in leaf tissue rings of *Psychotria capensis* and *Scolopia mundii* when their water content decreased. Some possible sources of error in the techniques employed will be mentioned.

Damage to tissues by cutting the tissue rings. Employing the technique outlined above it was found that the resistance of the mesophyll tissue of leaves of one species grown in the same locality was statistically constant. It followed that either the damage done by cutting the tissue rings was negligible or that the damage was always of the same consequence. If the latter was the case the values found for the resistance of the mesophyll tissues may have been too high or too low, but this could hardly affect the progressive increase in this resistance with decreasing water content.

Gradual clogging of the cut edges. This phenomenon was indeed observed with leaves of *Ficus natalensis* and *Carissa grandiflora* which contain latex. However, changes in the resistance of their mesophyll tissues were erratic and soon reached magnitudes which were beyond the limits of the instrument. If such tissue rings with clogged edges were trimmed with razor-sharp punches their resistances decreased considerably. On the other hand, if tissue rings of *Psychotria capensis* and *Scolopia mundii* with slight water deficits were trimmed in the same manner their resistances did *not* decrease appreciably. Microscopic observations of the cut edges also indicated that clogging occurred with some leaf tissues, but it could not be detected in those of *Psychotria capensis* and *Scolopia mundii.*

Fitting of tissue rings between washers. As the rings had to be fitted anew after each loss in weight, it is possible that not exactly the same conditions obtained for each reading. Washers and tissue rings fitted well and there was no overlap; however, with each of the species additional readings were taken without removing the tissue rings from the porometer, instead moving the whole porometer out of the constant moisture chamber. When measurements of the resistance had increased to a certain extent, the tissue rings were transferred to the aperiodic balance in order to determine their decrease in water content. This technique had the disadvantage that readings could be taken at only two different water content levels. Results so obtained were entered in Fig. 2 and fell all within the shaded area. This would suggest that the fitting of the tissue rings between the washers did not cause serious errors.

Compressibility of tissues. Increases in the resistance of mesophyll tissues were probably due to partial collapse of the internal air spaces when cells ceased to be fully turgid. It is known from work with leaf thickness change meters (Meidner, 1952) that the leaf thickness does change with changes in leaf water content. In addition, however, leaves may become more compressible when their water content decreases and the pressure of the porometer clamp may cause a measure of compression which would not occur in uninterfered leaves.

In order to test the effect of the clamp pressure on the gradually increasing resistance of mesophyll tissue rings with decreasing water content, a series of measurements was carried out in which the tissue rings were held on to the lower washer with a thin film of vaseline and on their upper surface a coverslip with a thin film of vaseline made an airtight joint. Clamp pressure was thus eliminated and resistances so measured were found to be up to 20 per cent. lower than those measured with a clamp. If weights of 20, 50, or 100 g. were placed on the vaselined coverslips variations in the measurements of resistances were obtained. However, the resistances still increased gradually when the tissue water content decreased whether or not a pressure was applied, and independent of the magnitude of the pressures applied.

Results of measurements of increases of the resistance of mesophyll tissue accompanying decreases in their water content may thus be magnified, but these increases would not seem to have been caused by the pressure of the clamp.

All porometer measurements involving a clamp pressure, whether of total resistance offered by leaves to viscous flow of air or of the resistance of the mesophyll tissue alone, may include a magnification of the real resistances.

CONCLUSIONS

Penman (1942) has shown that the mesophyll resistance varies with the stomatal conductivity owing to the variation in area of leaf tissue which is involved in offering resistance to viscous flow of air with each particular stomatal conductivity. Calculations of these variable mesophyll resistances involve complicated mathematics including Bessel functions.

From the results of measurements of the resistance of mesophyll tissues reported here it appears that this resistance varies also with the water content and the latter changes often with the stomatal conductivity. Therefore the separation of stomatal conductivities from measurements of total resistances offered by leaves to viscous flow of air is further complicated.

The increase in the mesophyll resistance accompanying decreases in leaf water content may constitute an additional factor affecting the water economy of plants. This may be indicated by the comparatively steep rise of the mesophyll resistance after the leaf water content had decreased beyond those values which occurred commonly in the field.

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Histochemical and Quantitative Observations on the Distribution of Galiosinase in the Shoots of Stellateae

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SUMMARY

1. A histochemical method for the detection of the specific glycosidase galiosinase in plant tissues is described.

2. A colorimetric method for the estimation of the enzyme is described.

3. The distribution of the enzyme in the shoots of 14 species of Stellateae, representing 5 genera, has been investigated.

4. In the leaves and stem galiosinase occurs in high concentrations in the cambium and in lower concentrations in the developing phloem and xylem.

5. In some parts of the stem near the base of the shoot and at the nodes the enzyme may also occur in the medulla and cortex.

6. When galiosinase occurs outside the cambium it may be accompanied by its specific substrates, the glucoside and primveroside of purpurin-3-carboxylic acid.

7. In the parts which accumulate the purpurin-3-carboxylic acid glycosides there is an approximately direct proportionality between the concentrations of enzyme and glycoside.

8. When kept in water for a few days the excised leaves and stems of some species produce large amounts of galiosinase.

9. The formation of enzyme in excised parts is closely followed by the accumulation of purpurin-3-carboxylic acid glycosides.

10. The bearing of these observations on the problem of the physiological role of glycosidases (glycoside synthesis, lignification, &c.) is discussed.

INTRODUCTION

HISTOCHEMICAL tests are an important aid to the understanding of the role of enzymes in the organism since they can provide a precise and detailed knowledge of the distribution of these catalysts in different organs, tissues, and cell structures. It is unfortunate that there are still so few of them applicable to plant tissues (see van Fleet, 1952). In the work to be described a simple histochemical test for the hydrolytic enzyme galiosinase (Hill and Richter, 1937) has been developed and applied to the study of its distribution in plants in the tribe Stellateae (Galieae) of the family Rubiaceae. Galiosinase hydrolyses the glycosides of purpurin-3-carboxylic acid, two of which have

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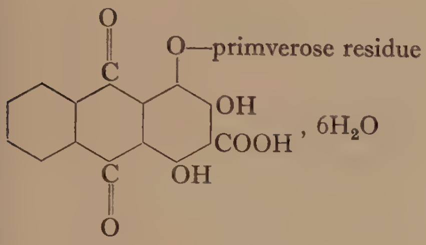
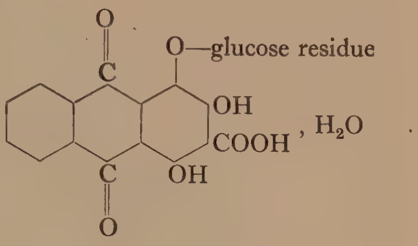
been isolated from different representatives of the tribe: galiosin, a primveroside (Hill and Richter, 1937) (I) and a monoglucoside (Hill and Trim, unpublished) (II):

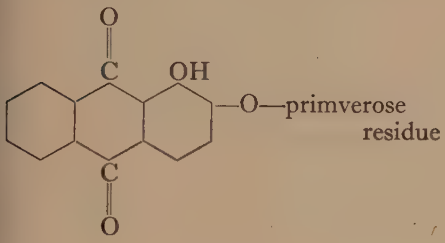


(I)

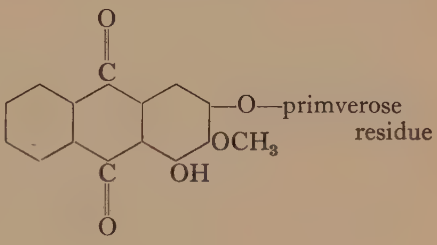


(II)

Galiosinase is a highly specific enzyme which selectively splits the glycosidic link of the two purpurin-3-carboxylic acid glycosides and has little action on other anthraquinone glycosides such as ruberythric acid (III) and rubiadin primveroside (IV), which are found in the roots of the same group of plants.



(III)



(IV)

The latter are hydrolysed by the enzyme erythrozyme, which occurs in the roots of all Rubiaceae so far tested (Hill and Richter, 1937). The complete separation of galiosinase and erythrozyme activities in enzyme preparations from the root of *Rubia tinctorum* (dyer's madder) (Hill and Trim, unpublished) has shown that the two activities are due to two distinct enzymes.

The chemical properties of both galiosinase and purpurin-3-carboxylic acid are ideally suited to histochemical studies of the enzyme. The two glycosides of purpurin-3-carboxylic acid dissolve in water to give yellow solutions, but the aglycone is insoluble in water at physiological pHs and has a very intense red colour which makes it easy to detect in very small quantities. The enzyme galiosinase although soluble in strong salt solutions is insoluble in water and dilute salt solutions. Consequently when sections of plants which contain galiosinase are treated with acetone and then soaked in aqueous solutions of either of the two purpurin-3-carboxylic acid glycosides, cells containing the enzyme become stained bright red owing to the deposition of purpurin-3-carboxylic acid at the site of enzyme action.

This simple method makes it possible to study the distribution of the enzyme in such detail that in most plant tissues cell-to-cell differences in enzyme activity are easily distinguished. Intracellular localization of the enzyme cannot be discerned. In applications of this method to the study of rubiaceous plants many of the observations were supplemented by the estimation of the enzyme activity in solutions obtained by extraction of the different tissues with strong salt solutions.

METHODS

The histochemical method. Uninjured cells containing galiosinase show no signs of engaging in hydrolytic action either on the purpurin-3-carboxylic acid glycosides which may be contained by the cells or on the same glycosides applied externally. However, any injury to these cells initiates this activity. For histochemical purposes the necessary destruction of the structural integrity of the cells in the plant sections is achieved by brief immersion in acetone which precipitates the enzyme *in situ* and renders it accessible to its substrates.

Freehand or microtome sections of fresh plant material, usually 200 μ in thickness, are soaked for 5 minutes in acetone and washed in water. Washing for a few seconds is sufficient, but much more prolonged immersion in water at this stage is not harmful. The washed sections are transferred to a buffered solution of galiosin of the following composition: 0·425 per cent. (w/w) galiosin, prepared by the unpublished method of Hill and Trim, in 0·1 M. Sørensen's citrate buffer pH 6·5 containing 0·1 M. $CaCl_2$. The Ca^{++} strengthens the fixation of the precipitated purpurin-3-carboxylic acid and almost completely eliminates the tendency of this substance to wash out of the sections when passing through the alcohols during the subsequent dehydration process. After incubation with the substrate at room temperature the sections are washed in water and those required to be kept are mounted in Canada balsam after the usual dehydration and clearing by passage through the alcohols and xylol. Preparations made in this way have been kept for more than a year without signs of deterioration.

It is necessary to be sure that the staining of acetone-treated sections immersed in galiosin is entirely due to enzymic action, that this occurs at the site of staining and is not due to the fixation of floating or diffusing purpurin-3-carboxylic acid arising from enzyme action at another site, and that the unstained cells in a section have retained their contents and have therefore remained unstained because of the absence of galiosinase.

The enzyme-catalysed nature of the staining is readily shown by treating sections containing enzyme with acid or heat before immersion in galiosin solution. When this is done, as is shown in Fig. 1 there is no staining.

The photographs in Fig. 2 show the effect of increasing the time of incubation on the development of staining in identical sections of the stem of *Rubia tinctorum*. If the enzyme is present at all, the staining reaction is quite rapid and it is usually possible to obtain a definite indication of the location of the enzyme in from 1 to 10 minutes' immersion in galiosin solution at room temperature. The sections in the illustration show no effects attributable to diffusion of purpurin-3-carboxylic acid in up to 20 minutes' staining. More

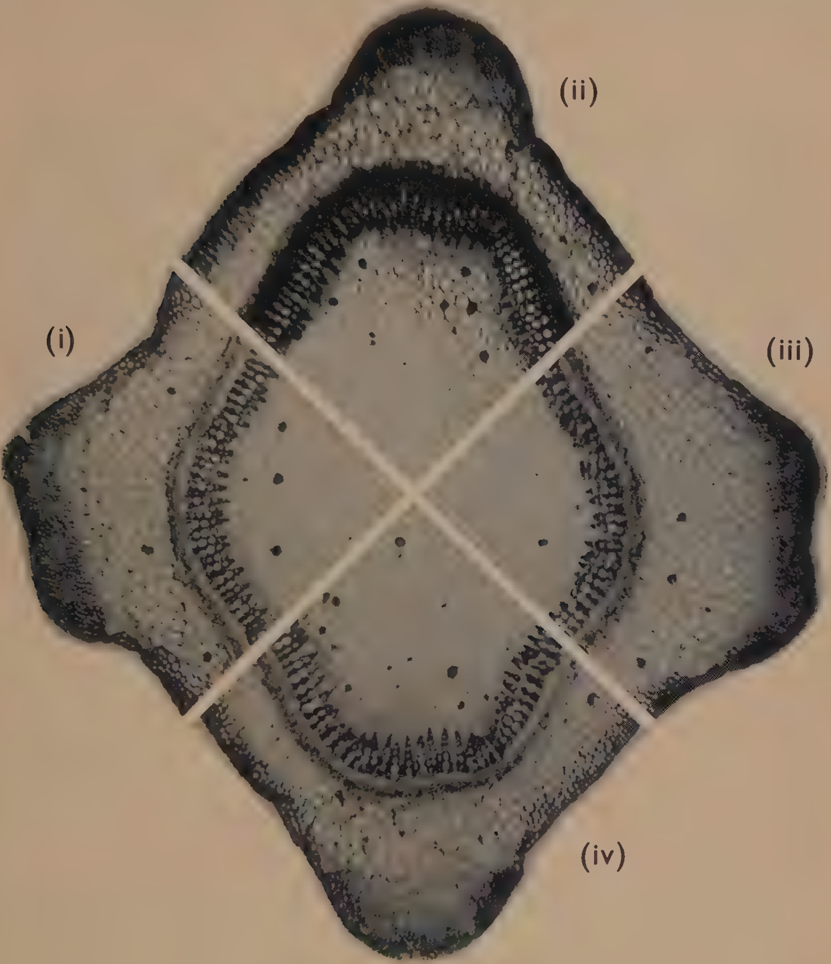


FIG. 1. Transverse section (200 μ) of internode of aerial stem of *R. tinctorum*: (i) unstained control, (ii) stained with galiosin, (iii) heated to 100° C. for 5 minutes before immersion in galiosin, (iv) immersed in 1 N. HCl for 5 minutes before immersion in galiosin.

prolonged immersion does not lead to any appreciable signs of the development of artifacts. Tissues containing enzyme and enzyme-free parts such as the stem cortex of *R. tinctorum* may be separated by dissection, the parts stained separately or extracted with 10 per cent. KCl solution, and the amount of enzyme estimated colorimetrically (see the following section). Such tests confirm the faithfulness of the picture of enzyme distribution obtained by the immersion method described above.

The estimation of galiosinase: Extraction of the enzyme. Fresh plant tissues are ground in a mortar chilled to −10° C. with acetone at the same temperature, using at least 10 volumes of acetone to each volume of tissue and changing the acetone three times. The residue is filtered off on a Buchner funnel with a thin layer of kieselguhr on the filter paper. The acetone powder thus obtained is allowed to stand for a time to permit the evaporation of the residual acetone and then stood for 30 minutes with 6·6 per cent KCl in M/9 Sørensen phosphate buffer pH 5·9 (solution *S*). The enzyme extract is squeezed through muslin and clarified by centrifugation. The extract remains active indefinitely if kept at −10° C.

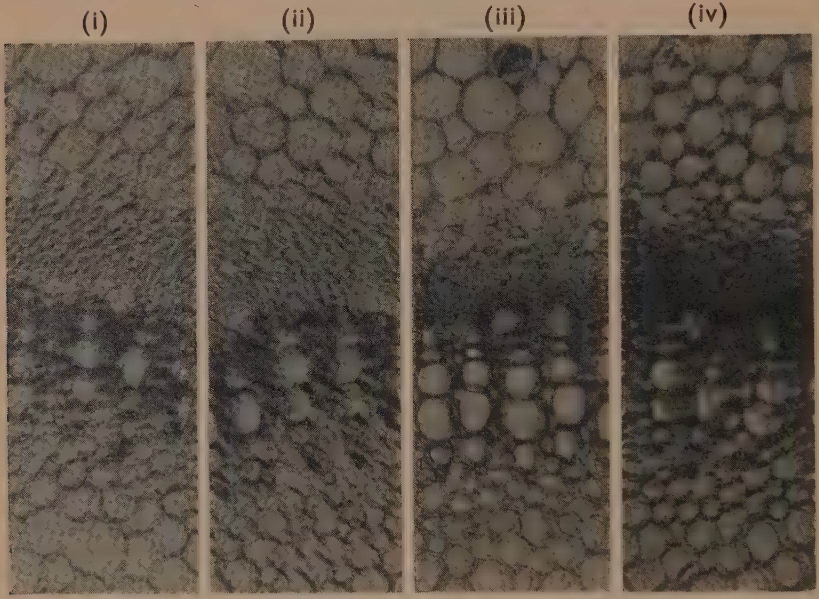


FIG. 2. Transverse sections (200 μ) of stem of *R. tinctorum* in the vascular region with different times of immersion: (i) 0 min., (ii) 2 min., (iii) 5 min., (vi) 20 min.

Estimation of the enzyme. The properties of the extracted enzyme are studied by following the production of purpurin-3-carboxylic acid colorimetrically. On the suggestion of Dr. G. Weber the water-insoluble pigment is solubilized by serum albumin (Armour fraction 5) which forms water-soluble compounds with insoluble organic acids such as anthraquinone carboxylic and sulphonic acids (Laurence, 1952). The enzyme reactions are conducted in solutions containing an excess of serum albumin. At the end of the experiment the reaction is stopped by acidification to pH 2·5 with dilute HCl and the required dilution for reading is made with 0·1 M. glycine buffer pH 2·5.

A calibration curve is constructed in the following way. A volume x ml. of 1·3 per cent. crystalline galiosin in water is added to a solution containing 0·4 ml. 10 per cent. serum albumin in solution *S* and 0·1 ml. of a strong solution of galiosinase in solution *S*. Hydrolysis of galiosin is allowed to go to completion and the enzyme is inactivated by the addition of 0·5 ml. 0·2 N. HCl; y ml. of 1·3 per cent. galiosin is then added so that $x+y = 0{\cdot}25$. The solution is diluted by the addition of 10 ml. 0·1 M. glycine buffer pH 2·5.

The curves 1 and 2 in Fig. 3 are the absorption spectra of the solutions when $x = 0{\cdot}25$ and $0{\cdot}0$ respectively measured in the Beckmann spectrophotometer after dilution 1:4 with glycine buffer pH 2·5. Fig. 3 shows that the solutions containing purpurin-3-carboxylic acid have an absorption band which reaches a maximum at 545 mμ, a wavelength at which solutions containing only galiosin have a very low absorption. This wavelength was chosen as convenient for the measurement of purpurin-3-carboxylic acid in the above solutions and the optical density at this wavelength was measured with a Unicam diffraction grating spectrophotometer. The band width for this instrument is about 35 mμ, so that absorption over the range 527–562 mμ (*A–B* in Fig. 3) is actually measured. The extinction is plotted against mg. purpurin-3-carboxylic acid in Fig. 4.

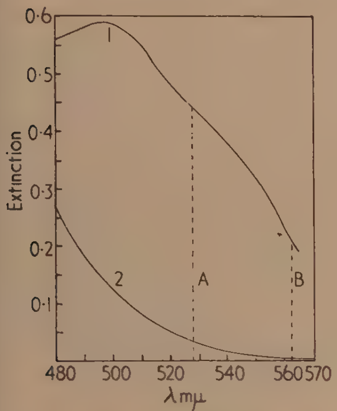


FIG. 3. Curve 1: Absorption spectrum of solution containing the standard quantity of crystalline galiosin, 3·25 mg., completely hydrolysed by galiosinase, i.e. 1·4 mg. purpurin-3-carboxylic acid, 0·25 ml. water, 0·4 ml. 10% serum albumin dissolved in solution *S*, 0·1 ml. solution *S*, 0·5 ml. 0·2 N. HCl, and 43·75 ml. glycine buffer pH 2·5. Curve 2: Absorption spectrum of the same solution without hydrolysis of the galiosin, i.e. 0·0 mg. purpurin-3-carboxylic acid.

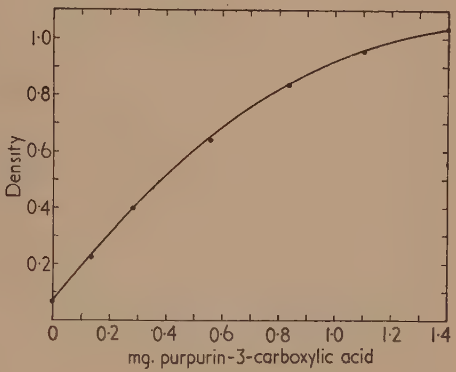


FIG. 4. The relation between optical density *d* measured in the Unicam diffraction grating spectrophotometer and mg. free purpurin-3-carboxylic acid in the presence of unhydrolysed galiosin remaining from 3·25 mg. of the hydrated glycoside; total volume 11·25 ml., comprising 0·25 ml. water, 0·4 ml. 10% serum albumin dissolved in solution *S*, 0·1 ml. solution *S*, 0·5 ml. 0·2 N. HCl, and 10 ml. glycine buffer pH 2·5.

Under these conditions the optimum activity of galiosinase is at pH 5·8 (Fig. 5) and the enzyme unit has been arbitrarily defined as the amount of enzyme which will produce 0·1 mg. purpurin-3-carboxylic acid in 15 minutes at 20° C. and pH 5·8 in a solution containing 0·5 ml. 8 per cent. albumin in solution *S* and 0·25 ml. 1·3 per cent. galiosin in water.

Under these conditions there is a linear relation between the amount of enzyme and the amount of purpurin-3-carboxylic acid produced in a given time. Plotting this relationship for different times a series of straight lines

(Fig. 6) is obtained making angles θ with the abscissa. Tan θ is plotted against time in Fig. 7. If under standard conditions a solution of galiosinase produces a quantity a mg. of purpurin-3-carboxylic acid in time b, then the amount of enzyme e is given by equation (1)

$$e = \frac{a}{\tan \theta_b}. \qquad (1)$$

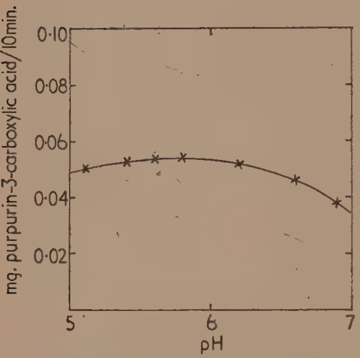


FIG. 5. Relation between the activity of galiosinase, in terms of mg. purpurin-3-carboxylic acid, produced under standard conditions at 20° C. and the pH of the solution.

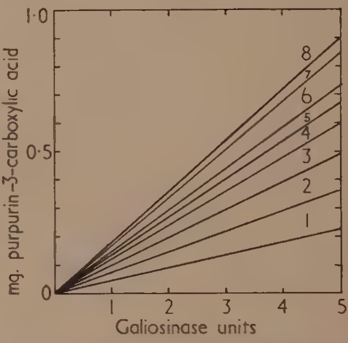


FIG. 6. Curves showing the relation between the quantity of purpurin-3-carboxylic acid produced under standard conditions and the amount of enzyme present. Times of incubation at 20° C.: 5 min., curve 1; 10 min., curve 2; 15 min., curve 3; 20 min., curve 4; 25 min., curve 5; 30 min., curve 6; 40 min., curve 7; 50 min., curve 8.

This method was intended to be used with galiosinase solutions obtained by extraction of the plant tissues with solution S with no further treatment other than clarification by centrifugation. Inhibitory organic substances such as phenols will have been removed by the acetone, and some of the mineral components will also have been removed at this stage. However, some of the inorganic material will remain in the acetone powder and act as a source of substances which might interfere with the activity of galiosinase. Among such substances Ca^{++}, Mg^{++}, and Mn^{++} are those most likely to be present and most likely to have an effect on the activity of the enzyme. Concentrations of these ions ranging from M/1,000 to M/20 were tested. Mg^{++} was found to have no action and Ca^{++} and Mn^{++} up to M/100 were also without effect. However, M/20 Ca^{++} and Mn^{++} had an inhibitory action (10 and 25 per cent. respectively). In both cases there was precipitation of phosphate. It may be concluded that the extraction procedure avoids the extraction of significantly inhibitory concentrations of Ca^{++} and $Mn.^{++}$ For in the first place it is improbable that the plant material would yield such large concentrations of either of these ions; and secondly, if it did, these concentrations would be reduced to an insignificant level during the extraction with phosphate buffer.

While the possibility of interference by uncontrolled factors has not been completely eliminated, it seems permissible to use this method for quantitative purposes, at least in the type of comparative experiments which are to be described.

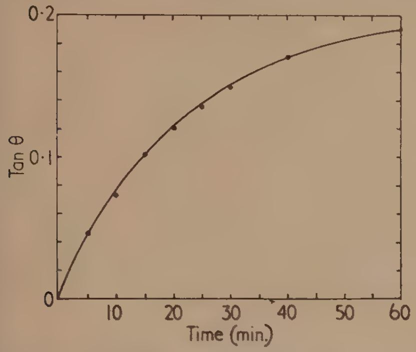


FIG. 7. Relation between the slopes of the curves in Fig. 6 and the time of incubation at 20° C.

The efficiency of enzyme extraction. Galiosinase is extracted from the fresh acetone powder with solution *S* added in the proportion of 3 ml. solution *S* for every 1 g. fresh weight of plant material. Most of the enzyme is removed by three successive extractions and it is found convenient to use a combination of the first two extracts in quantitative studies. Table I shows that about 75 per cent. of the total enzyme obtained in the first three extracts is present in the first while about 95 per cent. of it is found in the first two extracts combined.

TABLE I

The extraction of galiosinase from fresh stem tissue of R. tinctorum

Fresh wt. of sample (g.)	Units (*e*) of galiosinase extracted					
	a first extract	*b* second extract	*c* third extract	*a*+*b*+*c*	*a* as % of (*a*+*b*+*c*)	*a*+*b* as % of (*a*+*b*+*c*)
2·9	47·5	13·6	3·4	63·5	74	95
2·4	34	8·5	3·4	45·9	74	90
1·05	377	89	23	489	77	95
0·76	190	40·5	8·6	239	80	97
0·53	106	26	5	137	77	96
0·67	184	48·5	10·3	242·7	76	96
0·56	140	54·6	17·9	212·5	66	92
0·51	39	8·9	2·0	49·9	78	96
0·57	53	10·9	2·0	65·9	80	97

THE DISTRIBUTION OF GALIOSIN IN THE TRIBE STELLATEAE

Hill and Richter (1937) characterized purpurin-3-carboxylic acid as a specific product of the tribe Stellateae because it occurs constantly within this group and has not been detected in any other plants. They examined 23 species, including representatives of 5 genera, and found glycosides of purpurin-3-carboxylic acid in the roots of all of them.

In the majority, but apparently not all, of the plants in the tribe Stellateae anthraquinone pigment accumulation is very largely confined to the roots and the short basal internodes of the shoot. In the present work and that of Hill and Richter (1937) the shoots of *R. tinctorum* have been examined in detail and it has been found that the pigmented parts of the young shoot contain glycosides of purpurin-3-carboxylic acid but no detectable quantity of any other known anthraquinone derivative, whereas the root usually contains ruberythric acid and/or rubiadin primveroside and probably other anthraquinone pigments in addition to purpurin-3-carboxylic acid glycosides. Analysis of shoot extracts by filter-paper chromatography has shown that the stem pigment is predominantly the glucoside of purpurin-3-carboxylic acid whereas in the root there is a much higher proportion of the primveroside. The R_F values for these components were identical with those of authentic specimens of the two glycosides.

THE DISTRIBUTION OF GALIOSINASE IN THE TRIBE STELLATEAE

No systematic study of the roots of the plants in Stellateae has been made for the presence of galiosinase, but in the present work 14 of the species found by Hill and Richter (1937) to contain purpurin-3-carboxylic acid derivatives in the roots have been found to have galiosinase in the aerial parts. These species contain representatives of all five genera investigated by Hill and Richter (1937) and are as follows: *Rubia tinctorum*, *R. peregrina*, *Crucianella stylosa*, *Asperula odorata*, *Sherardia arvensis*, *Galium boreale*, *G. mollugo*, *G. rubiodes*, *G. corrugifolium*, *G. sylvaticum*, *G. verum*, *G. aparine*, *G. cinerarium*, *G. erectum*.

Photographs illustrating the distribution of the enzyme are provided in Figs. 8, 9, and 10. A systematic quantitative study of the distribution of galiosinase in relation to the growth of the shoot has not been made but such observations as have been made are sufficient to permit certain generalizations which provide the basis for a deeper investigation into the physiological role of galiosinase and its substrates. There is a common pattern of galiosinase distribution in all the plants which have been investigated and the variations in the pattern which do occur are more closely connected with the stage of growth of the plant than with taxonomic differences.

There are four main features of the distribution of galiosinase in the stem:

1. If the enzyme is present in a section it is always found in the cambium and phloem although in most material it does not extend beyond this region.
2. In parts of the plant in the soft, elongation stage of growth the enzyme is weaker than in the fully grown parts.
3. In more mature parts of the stem, e.g. the base of a fully grown shoot, the enzyme is often found in both the cortex and the medulla.
4. The occurrence of the enzyme in the cortex and the medulla is more likely to occur at and very near to the nodes than in any other part of the stem and foliage.

In all the species listed above, stems well advanced in the elongation stage were found to contain galiosinase confined to the phloem and cambium as illustrated by the photographs in Figs. 8, 9, and 10, which are representative. The location and relative amount of enzyme may be seen by comparison of the stained half-section with the unstained half-section mounted below it.

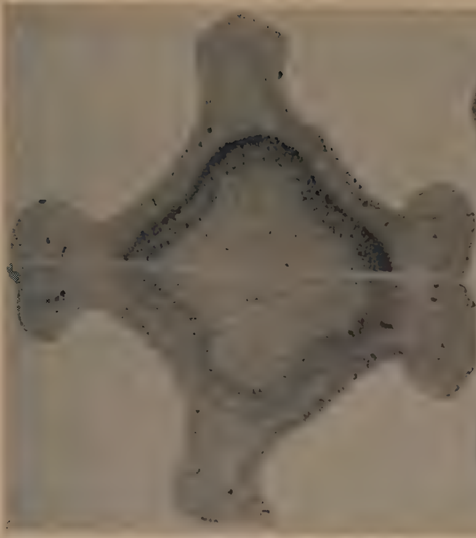


Fig. 8.

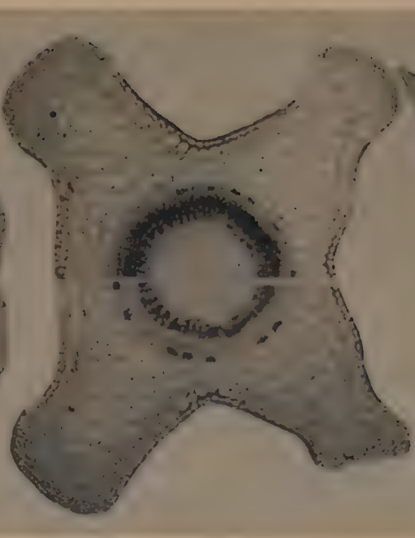


Fig. 9.

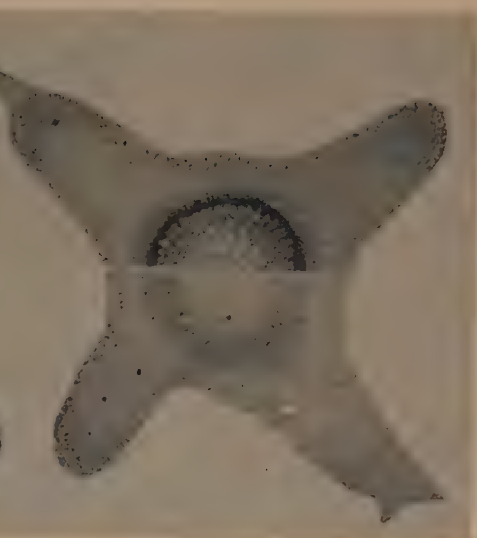


Fig. 10.

Fig. 8. Transverse section (200 μ) of internode of *Crucianella stylosa*: upper half stained with galiosin, lower half unstained control.
Fig. 9. Transverse section (200 μ) of internode of *Asperula odorata*: upper half stained with galiosin, lower half unstained control.
Fig. 10. Transverse section (200 μ) of internode of *Rubia peregrina*: upper half stained with galiosin, lower half unstained control.

The dark areas common to the stained and unstained sections are the relatively opaque parts such as the xylem and various concretions and crystalline deposits. The additional dark areas in the stained half-sections are the red-stained region developed by galiosinase activity in those parts.

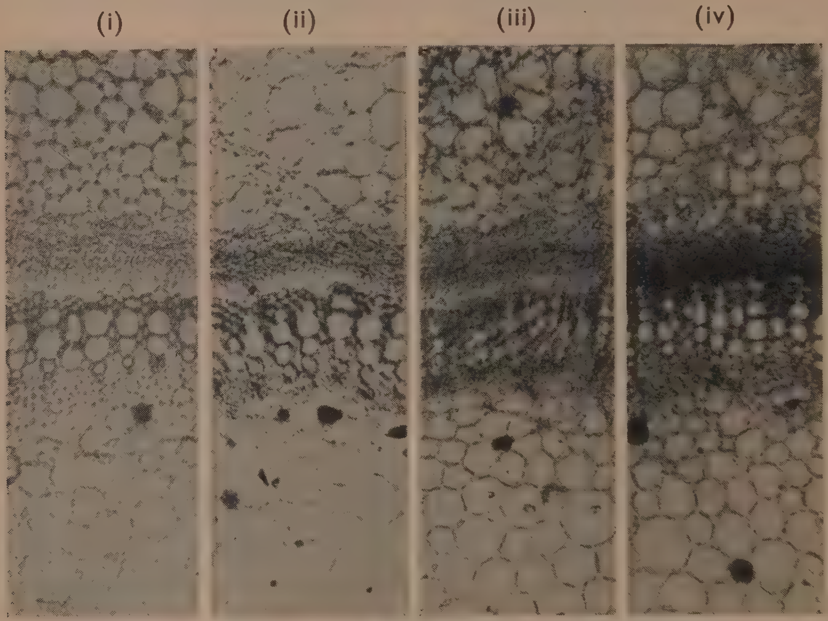


FIG. 11. Transverse section of stem of *R. tinctorum*: (i) unstained control; (ii) enzyme destroyed by acid, then section immersed for 20 min. in a solution generating purpurin-3-carboxylic acid (a galiosin solution containing a small amount of galiosinase); (iii) enzyme destroyed with acid, then section immersed for 20 min. in a solution of galiosin containing more galiosinase than in (ii); (iv) section with active galiosinase immersed in galiosin solution for 20 min.

The most striking feature of the examples illustrated is the outstandingly high activity of the enzyme in the cambium. A detailed examination of all the very numerous preparations made from green parts showed that this occurs in all the plants examined. The preparations include longitudinal and transverse sections of internodes from all levels of the well-developed shoots of the 14 species listed on p. 108, many sections of nodes, leaves, and young shoots. Observations were made in three consecutive seasons.

In addition the enzyme is present, though at a lower level of activity, in the other living parts of the vascular bundles at all stages of growth of the shoot. In the phloem it occurs mainly in the inner region and when present in the xylem it is found only in the narrow band of young xylem cells and is absent in the older dead vessels.

Purpurin-3-carboxylic acid in colloidal suspension deposits preferentially on thick-walled tissues (Fig. 11 (ii) and (iii)) so that it would be unprofitable to press the analysis of the histochemical results too far. Nevertheless it seems significant that in a detailed examination of sections of *R. tinctorum*

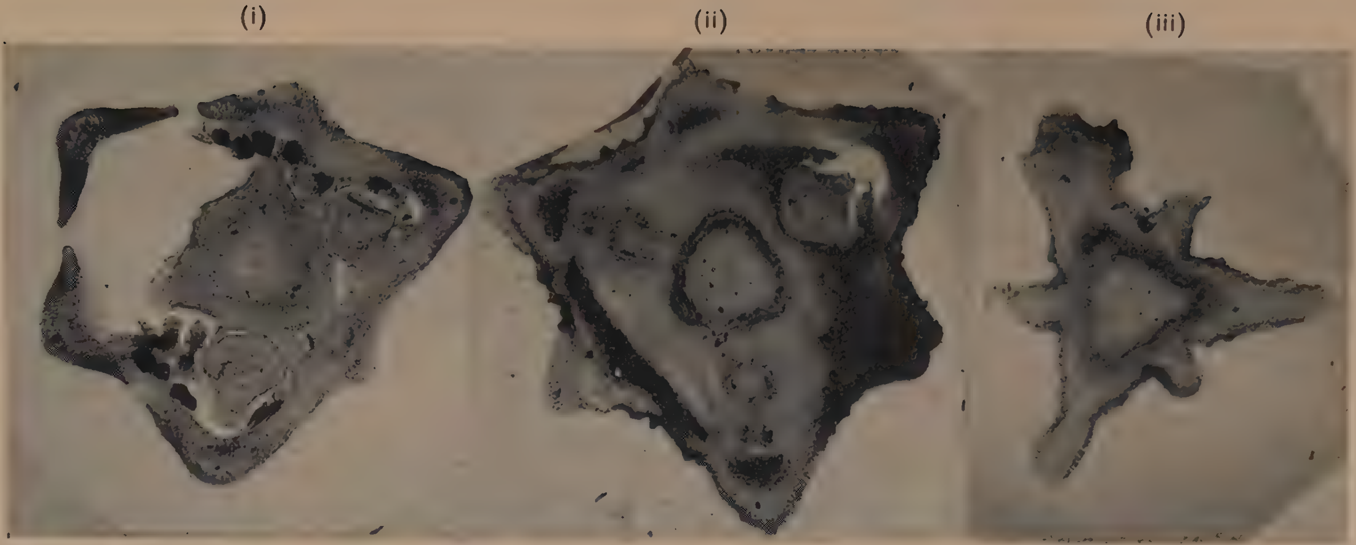


FIG. 12. Transverse sections (100 μ) from the growing tip of the shoot of *R. tinctorum* showing the localization of galiosinase in the axillary glands, parts of the embryonic leaves but not in the vascular tissues, (i) is 0·2 mm. from the tip, (ii) 1·4 mm., and (iii) 1·8 mm.

it was found that the enzyme is exceptionally active immediately round the large vessels which may be seen developing in the young parts of the xylem. With due consideration for the possibility of the formation of artifacts, this observation must be connected with a number of others with which it is consistent, all indicating the association of high galiosinase activity with rapidly differentiating cells undergoing lignification.

The sections in Fig. 12 are from the growing tip of *R. tinctorum* and are composed of cells in the meristematic and early elongation stages. The first 2 mm. of the shoot apex were cut into a series of 100 μ sections and tested for galiosinase. The top section (i) was from a level about 0·2 mm. from the tip; section (ii) was from a level about 1·4 mm. below the tip, and section (iii) about 1·8 mm. below. Galiosinase was entirely absent from the developing vascular tissues of stems but very strong in some parts of the embryonic leaves (ii) and in groups of glands found in the axils of the leaves (ii) and (iii). A comparison of sections from stems and leaves in the elongation stage shows that the enzyme is much more active in the vascular tissues once the part has passed through the elongation stage. Histochemical observations alone may not be considered an adequate basis for this conclusion. However, there are numerous quantitative observations in support of it and a selection of measurements on leaves of *R. tinctorum* showed that there is at least a twofold difference between the enzyme content of elongating and fully developed leaves taken from adjacent whorls of leaves (Table II).

TABLE II

Galiosinase in leaves of *R. tinctorum*

Shoot	Length of internode immediately below leaf (cm.)	Stage of growth	Galiosinase units per gm. dry weight
1	5	Elongation	175
	8	Fully grown	345
	7	Fully grown	420
2	5	Elongation	85
	12	Fully grown	181
3	5	Elongation	108
	12	Fully grown	270

In common with other plants, members of Stellateae (Trim, 1952) undergo certain sharp changes of metabolism in their young parts as they pass from the elongation stage into the fully grown stage. In Stellateae one manifestation of this is the cessation of the accumulation of the characteristic glucoside asperuloside (Trim, 1951) and the onset of its removal either by metabolism or translocation or by a combination of both processes. The large increase in galiosinase activity in the cambium and nearby cells, also closely linked with the change of the stage of growth, appears to be an enzymic aspect of the same general process. The appearance of galiosinase runs parallel with lignification.

Tests on seedlings of *Galium aparine* provided further data showing the close parallel between galiosinase formation and lignification. Seeds of *Galium aparine* were germinated in the dark on moist filter paper and the developing seedlings tested at intervals for galiosinase by the histochemical method. The results are recorded in Table III.

In addition it appears significant that all the plants examined had a higher activity of the enzyme in the older more woody parts. The values for the phloem and cambium in *Galium mollugo* are typical (Table III).

TABLE III

The localization of galiosinase in seedlings of Galium aparine

Time of germination (days)	Galiosinase in plant organ		
	Cotyledons	Hypocotyl	Radicle
7	Trace	—	—
10	+ in vascular bundles	—	+ in vascular bundles
13	+ in vascular bundles	+ in vascular bundles	+ in vascular bundles
48	+ in cambium and phloem	+ in cambium and phloem	+ in cambium and phloem

Histochemical tests for galiosinase at the base of internodes in shoots of Galium mollugo

Internode	Length (cm.)	Galiosinase in three parts of stem			Minimum time for definite staining (min.)
		cortex	vasc.	medulla	
1 (top of shoot)	1	—	+	—	10
2	2	—	+	—	10
3	4	—	+	—	10
4	10	—	++	—	10
5	11	—	++	—	10
6	13	+	+++	++	—
7	12	++	+++	+++	0·5
8	11	+++	+++++	+++	0·5
9	8	+++++	+++++	+++++	0·5
10	5	+++++	+++++	+++++	0·5

Some special features of the nodes. In some species the nodes and the internodal parts close to them are in striking contrast to the other parts of the stem. For in these parts galiosinase was found in cells outside the vascular region.

Galium mollugo provides a specially interesting example of this phenomenon. Tests on the mature shoot just coming into flower show that galiosinase is very active in a group of sharply demarcated cells in the swellings which develop at the base of the internodes in the more mature parts of the shoots. There is, however, no formation of anthraquinone glycosides in these parts. The swellings appear to have a function analogous to the pulvini of leaves. They remain soft when the rest of the internode has hardened and enable the trailing stem to bend itself so as to present its new growth to the light. This phenomenon is illustrated in Fig. 13. When there is no swelling of the cortex there is no enzyme in the medulla and cortex in this region. The swelling is not always radially symmetrical about the stele. When this occurs the enzyme only appears in the cortex on the side of the stem which is swollen and is absent from the side which is not swollen.

In the swollen part all the cells of the cortex contain galiosinase and the rate of staining shows that the amount of enzyme is high and of the order which is found in the phloem region. The stem is hollow, except at the nodes where a plug of pith occurs and in very young internodes. Galiosinase is strong and uniformly distributed in this medullary region precisely at the level of the swollen cortical layer. The plug of pith extends into the internode below, but scarcely any galiosinase may be found in that part of it.

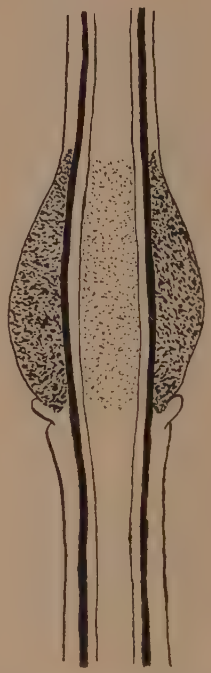


FIG. 13. Longitudinal section through the nodal region of *Galium mollugo* at the stage when swelling has occurred above the node. Galiosinase in the cambium is shown by a heavy black line; in the medulla and cortex it is shown by stippling.

Some details of the shoot of Rubia tinctorum. The underground part of the stem of *Rubia tinctorum*, unlike its upper part, but like the root, contains galiosinase and purpurin-3-carboxylic acid glycosides in considerable quantities. Sections cut from this part of the shoot show that the enzyme and the glycosides are uniformly distributed throughout all the cells. In the green part of the stem galiosinase is confined to the vascular tissues, as already described, and the purpurin-3-carboxylic acid glycosides are not present at all. In between these two well-defined parts there is a transitional section of the stem in which the glycosides and the enzyme are still found together. Moving along the stem on a vertical axis the amount of enzyme external to the vascular tissues decreases rapidly with the distance from the root and also with the perpendicular distance from the vertical axis, as is shown in Fig. 14. The occurrence of the purpurin-3-carboxylic acid glycosides conforms to the same pattern, although it does not coincide exactly with galiosinase which extends beyond the range of the glycosides.

The following experimental data show the precise nature of the change over the transitional section of the stem. A shoot which has just come through the ground and is showing the first whorl of leaves, still in the elongation stage of growth, has four internodes above the ground : *A*, *B*, *C*, and *D*, of which *D* is the most apical. The distribution of the glycosides and galiosinase in these internodes is described in Table IV.

The distribution of the enzyme is shown in the form of a drawing in Fig. 14 and photographs of stained sections are reproduced in Fig. 15 in which photographs of sections of higher internodes on the same stem are included for comparison.

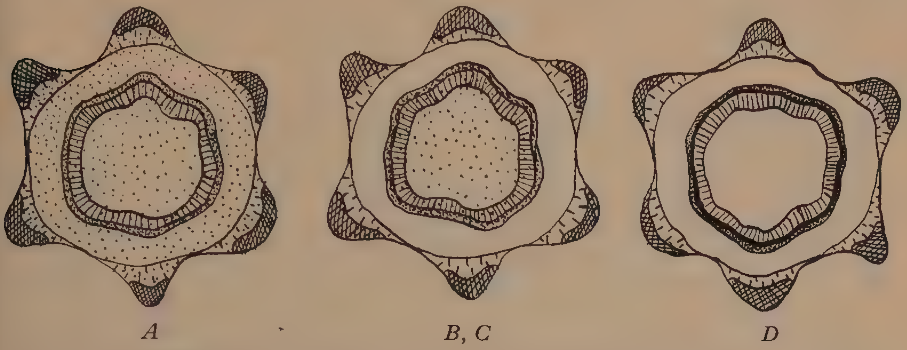


FIG. 14. Transverse sections of three successive basal internodes of the stem of *R. tinctorum*, *A* being the lowest. The presence of enzyme is shown by stippling.

These qualitative results are confirmed by quantitative determinations of the enzyme and the glycosides in successive internodes corresponding to *A*, *B*, and *C*. The enzyme was extracted from acetone powders of the fresh tissues and the acetone filtrate, containing all the anthraquinone pigments, was reduced to a small volume and the water precipitated by the addition of petrol ether. The chlorophyll and other fatty substances remained in the petrol ether, the anthraquinone glycosides passing into the water. The solution was made up to a definite volume with water and used for the estimation of the anthraquinone glycosides and their identification by filter-paper chromatography. Using three different solvent mixtures: butanol-acetic acid-water, 40:10:50 v/v/v; pentanol-ethanol-water and propanol-water 80:20 v/v, only two of the known anthraquinone glycosides were detected, purpurin-3-carboxylic acid primveroside and purpurin-3-carboxylic acid glucoside. The two components run at the same rate as authentic specimens with the glucoside in considerably higher proportion in each case. The total purpurin-3-carboxylic acid in each extract was measured by hydrolysing a sample with

TABLE IV

Galiosinase and purpurin-3-carboxylic acid glycosides in young shoots of R. tinctorum

	Galiosin and purpurin-3-carboxylic acid Glucoside			Galiosinase		
Part Node	Cortex	Phloem	Medulla	Cortex	Phloem	Medulla
D	—	—	—	—	+	—
C	—	—	—	—	+	+
B	—	—	+	—	+	+
A	+	+	+	+	+	+

N. HCl at 50° C., extracting the purpurin-3-carboxylic acid with toluene and measuring the extinction of the solution at 520 mμ with a Hilger photoelectric absorptiometer using an Ilford filter No. 624.

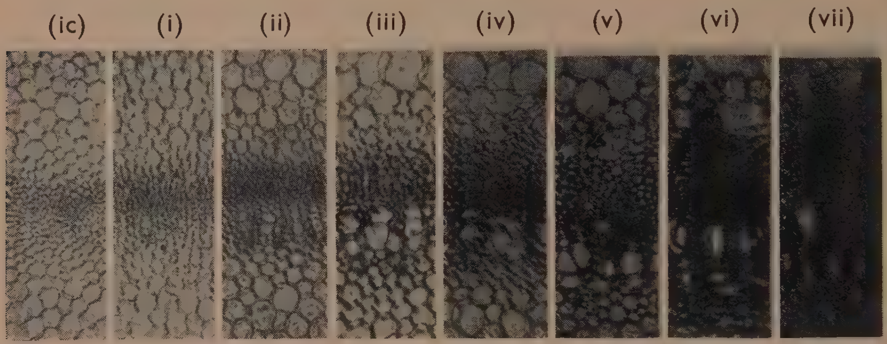


FIG. 15. Stained transverse sections (200 μ) of successive internodes of *R. tinctorum* numbered from the growing tip (i) downwards to the base (vii). (ic) is the unstained control of (i).

The concentrations of enzyme and purpurin-3-carboxylic acid in the different internodes are recorded in Table V. The distribution of the enzyme in the upper parts of the stem has already been discussed in the previous section and is illustrated in Fig. 15. At a later stage of development of the shoot purpurin-3-carboxylic acid glycosides appear in some parts of the stem, particularly at and near the nodes and, to a lesser degree, in the medulla of the internodes. The glycosides are always accompanied by galiosinase, but sometimes only the enzyme is present. There is some difficulty in establishing that in the growing shoot both glycosides and galiosinase arise in tissues in which they were not originally present. However, it can be very easily shown that this does happen in excised stems and leaves when they are kept in water for a few days.

TABLE V

Purpurin-3-carboxylic and galiosinase in young shoots of R. tinctorum

Shoot	Internode	Length (cm.)	Galiosinase/ g. wet wt.	urpurin-3-carboxylic (mg./g. wet wt.)	Enzyme units/mgs. aglycone
1	1	4	274	0·583	470
	2	2·6	235	0·707	330
	3	3·0	83·5	0·10	830
2	1	4	295	0·66	450
	2	2·6	172	0·575	300
	3	3·0	16·5	0·02	825
3	1	2	583	0·693	803
	2	2	323	1·0	323
	3	1·5	46	0·0	∞
4	1		730	0·73	1,000
	2		476	0·555	860
	3		68	0·02	3,400

The production of galiosinase and purpurin-3-carboxylic acid glycosides in excised parts of some members of Stellateae. Pieces of stem of several species of Stellateae kept alive in tap-water have been shown to generate large quantities of galiosinase, and in two selected plants, *R. tinctorum* and *Galium aparine*, purpurin-3-carboxylic acid glycosides have been shown to accumulate. The development of galiosinase in the stem of *R. tinctorum* is illustrated by the photographs in Fig. 16; an easily detected increase of enzyme occurs within 24 hours.

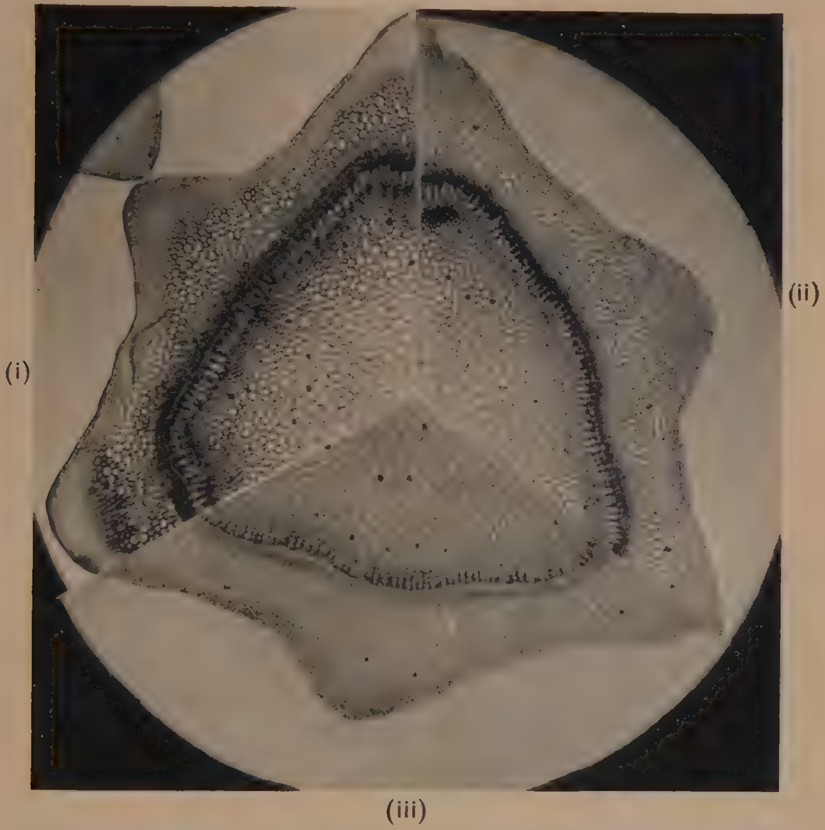


FIG. 16. Transverse sections (200 μ) of the stem of *R. tinctorum*: (i) section from an internode kept in water for 4 days before staining for 20 min. in galiosin solution; (ii) comparable fresh section stained for 20 min.; (iii) unstained control.

In a number of cases the production of enzyme has been followed quantitatively. Figs. 17 and 18 show the production of the enzyme by internodes of *Galium aparine* and *Rubia tinctorum* (cortex only), both of which, when fresh, contain so little enzyme that it cannot be detected by the very sensitive histochemical test.

Parts of the stem, which contain the purpurin-3-carboxylic acid glycosides when fresh, show no increase in galiosinase or glycosides when cut and kept in water. This may be demonstrated by splitting such internodes into two parts longitudinally, extracting the one part when fresh and the other after keeping for several days and comparing the amount of enzyme and glycoside.

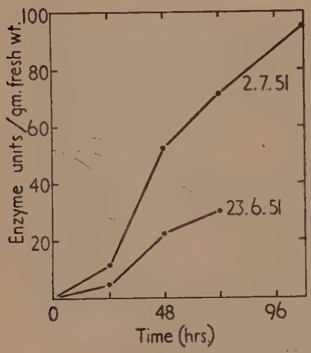


FIG. 17. Curve showing the production of galiosinase by internodes of *Galium aparine* kept in water at room temperature.

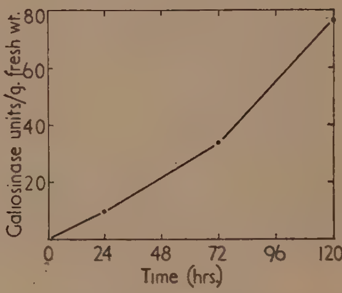


FIG. 18. Curve showing the production of galiosinase by the cortex of internodes of *R. tinctorum* kept in water at room temperature.

On the other hand, parts which contain little or no glycoside when fresh produce a large amount of enzyme over the same period (Table VI*a*).

A large number of tests, some of which were quantitative, showed that the property of developing galiosinase and purpurin-3-carboxylic acid glycosides when detached from the main part of the plant is common to *all* green tissues from an early stage of differentiation onwards (in *R. tinctorum*; other plants have not been investigated in detail).

Values for the production of the enzyme in the leaves of *R. tinctorum* are given in Table VI*b* and *c*, and photographs (Fig. 19) show that it develops in all cells.

Histochemical tests show that the enzyme is produced by both the cortex and the stele independently. The quantitative data in Table VI*b* show that the enzyme production occurs at the same rate in the two parts when separated from one another.

Excised parts of six other species were tested. Five species (Table VI*d*) behaved similarly to *R. tinctorum*, but the sixth (*Asperula odorata*) showed no production of galiosinase over a period of 3 days. Values for the production of galiosinase by *Galium aparine* have already been recorded in Fig. 17.

Analysis of excised stems and leaves of *R. tinctorum* and *Galium aparine* showed that the appearance of galiosinase is closely followed by the accumulation of the two glycosides of purpurin-3-carboxylic acid. The proportions of the two glycosides are comparable to those found in the glycoside-producing parts of the normal shoot, i.e. the ratio of the glucoside to the primveroside is high.

The glycosides were identified chromatographically on small samples of the extracted glycoside and the remainder was hydrolysed with dilute HCl and the purpurin-3-carboxylic acid extracted into toluene. Brown impurities removed by extracting the carboxylic acid into dilute $NaHCO_3$ and then returning it to toluene after acidification. The operation was repeated several times. The absorption spectrum of the purified pigment was measured in the Beckmann spectrophotometer and the yields calculated from the extinction at 527 mμ ($\beta = 2{\cdot}14 \times 10^{-7}$ cm.^{2}/mol.).

TABLE VI

The production of galiosinase and purpurin-3-carboxylic acid glycosides in excised parts of species in Stellateae

Plant and part		Fresh part		Time of keeping		After keeping	
		galiosinase (units/g. fresh wt.)	purpurin-3-carboxylic acid (μg/g. fresh wt.)	Days	Hrs.	galiosinase (units/g. fresh wt.)	purpurin-3-carboxylic acid (μg/g. fresh wt.)
(*a*)							
R. tinctorum							
Basal internodes of young shoots	1	583	730	5	12	466	693
	2	323	1,000	5	12	312	420
	3	730	730	5	12	460	693
	4	476	555	5	12	400	500
	5	46	0·0	5	12	260	140
(*b*)							
R. tinctorum							
Cortex		0	—	3	0	58·5	—
Stele		20·6	—	3	0	60·4	—
Leaves (*a*)*		13	—	4	0	288	—
Leaves (*b*)*		29	—	4	0	232	—
(*c*)							
R. tinctorum							
Leaves	1	37·5	0·0	1	19	179	1·0
	2	41·1	0·0	1	19	106	1·0
	3	27·2	0·0	1	19	80	1·0
Stem	1	14·9	0·0	1	19	61	1·0
	2	13·9	0·0	1	19	109	2·0
Leaves	1	—	—	3	17	313	9·0
	2	—	—	3	17	167	11·0
	3	—	—	3	17	220	4·0
Stem	1	—	—	3	17	150	12·0
	2	—	—	3	17	260	43·0
(*d*)							
G. mollugo							
Stem		13·4	—	9	0	103	—
G. erectum							
Stem		7	—	9	0	38	—
G. aparine							
Stem		0·0	—	4	0	55	—
G. aparine							
Leaves		7	—	4	0	50	—
Crucianella stylosa							
Stem		38	—	5	0	61	—
C. stylosa							
Leaves		23	—	5	0	37	—
A. odorata							
Leaves		0·0	—	3	0	0·0	—

* Leaves (*a*) last whorl in elongation stage. Leaves (*b*) first whorl of fully grown leaves.

The figures in Table VI*c* show the increase in galiosinase and purpurin-3-carboxylic acid in excised leaves and internodes from the upper part of mature shoots of *R. tinctorum*. Each sample comprises the corresponding parts from ten shoots.

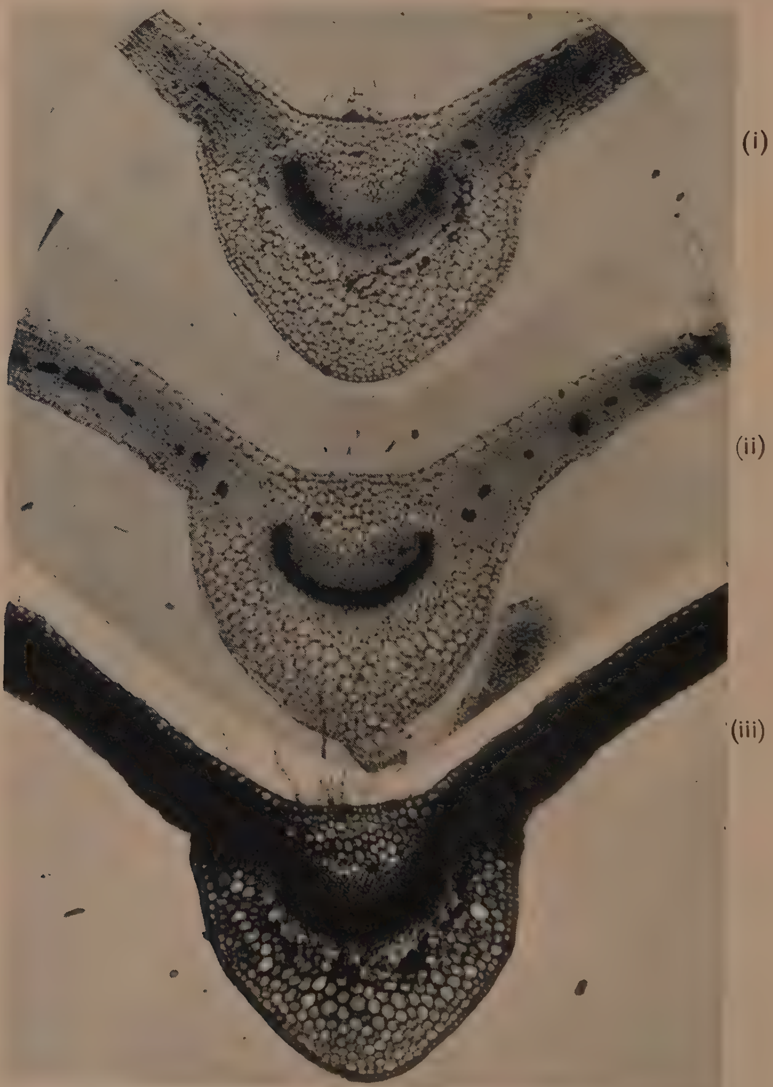


FIG. 19. Transverse sections of leaves of *R. tinctorum*: (i) fresh leaf, unstained control; (ii) fresh leaf stained with galiosin for 20 min.; (iii) leaf after keeping in water for 4 days stained with galiosin for 20 min.

The stems of *Galium aparine* also produce the two purpurin-3-carboxylic acid glycosides when kept in water. Mature internodes of this plant were kept for 28 days. During this time they lost their chlorophyll and acquired a golden colour due to the accumulation of the purpurin-3-carboxylic acid pigments. At the end of the 28 days the internodes contained 117 enzyme units per g. wet weight and 0·027 mg. purpurin-3-carboxylic acid per g. wet weight. The characteristic absorption spectrum of the aglycone obtained by mild acid hydrolysis of the golden pigment, plotted in Fig. 20, curve 3, is identical with that of the authentic sample (curve 1 in the same figure). Curve 2 is the spectrum of purpurin-3-carboxylic acid produced by the cortex of *Rubia tinctorum* (1·7 g. of fresh cortex produced 0·05 mg. of pigment in 24 days).

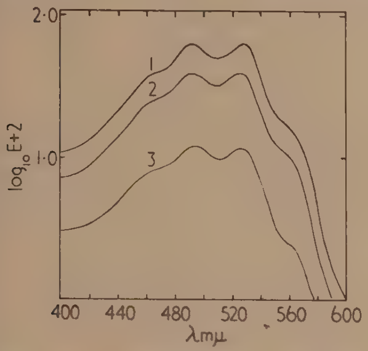


FIG. 20. Characteristic absorption spectra of purpurin-3-carboxylic acid: (1) obtained from purified authentic galiosin; (2) obtained from the glycosides produced by excised cortex of *R. tinctorum*; (3) obtained from the glycosides produced by excised internodes of *Galium aparine*.

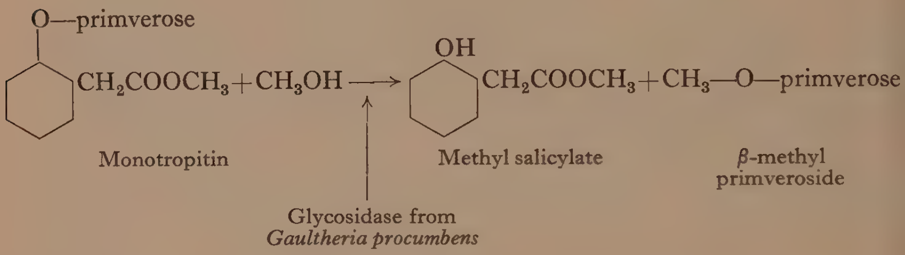
DISCUSSION

The above observations on a specific glycosidase and its natural substrates lead to a number of conclusions which have a bearing upon certain aspects of plant physiology.

There is a striking correlation between the accumulation of the two anthraquinone carboxylic acid glycosides and the presence of galiosinase in the cells concerned. Under both natural and artificial conditions the amount of glycoside accumulated, measured in terms of aglycone concentration, bears an approximately direct proportionality to the amount of enzyme present. The glycosides do not accumulate at all where there is no enzyme. On the other hand, however, the enzyme does occur in some parts of the shoot in the absence of detectable quantities of glycoside. These are the cambial region of the green parts, all cell layers in the nodes of some species, and a narrow zone at the base of the shoot (studied only in *R. tinctorum*) between the lowest part which contains both the enzyme and its substrate in all cells and the upper green part, which contains only enzyme confined to the region of the cambium.

It appears that in any particular cell the accumulation of the two purpurin-3-carboxylic acid glycosides follows upon the production of the enzyme by the cell. However, the absolute concentration of the glycosides must be greatly influenced by other factors for it to be possible to explain the extreme variations of the enzyme:substrate ratio which have been encountered. In spite of appearances it still remains possible that the production of the enzyme is an adaptive response to perhaps trace quantities of the substrate.

These observations provide sufficient ground to speculate upon the participation of galiosinase in the biosynthesis of one or both of the glycosides which it hydrolyses *in vitro*. *In vitro* observations on other glycosidases have shown that they can act in the synthetic sense under certain conditions (Bourquelot and Bridel, 1914). If the concentration of water in the system is reduced by the addition of alcohol or acetone, emulsin can be induced to act in the synthetic sense and in this way a number of alkyl β-glycosides have been synthesized, but no enzymic syntheses of phenol glycosides have been reported. Under these conditions a substantial degree of synthesis is only achieved in the presence of a high concentration of the organic solvent. However, synthesis in the presence of much lower concentrations of alcohol are obtained with certain glycosidase preparations (Rabaté, 1937) by a transglycosidation reaction in which sugar is transferred from glycosidic combination with a phenol to glycosidic combination with an alcohol, e.g.


O—primverose
$CH_2COOCH_3 + CH_3OH \longrightarrow$
OH
$CH_2COOCH_3 + CH_3—O—$primverose
Monotropitin
Methyl salicylate
β-methyl primveroside
Glycosidase from *Gaultheria procumbens*

In the presence of as little as 1–2 per cent. of methanol this reaction results in a 27 per cent. conversion of the phenol-linked primverose to methyl primveroside. As with the Bourquelot syntheses, all the synthetic products which have been obtained by this reaction are glycosides of alcohols.

It is a striking observation that in all the species examined high galiosinase activity is associated with the cambium especially in parts of the plant in which the phloem is fully differentiated and lignification is proceeding. Only one exception was found, *Galium aparine*, in which the fully grown upper parts of the fresh stem have no galiosinase. The distribution of the enzyme in the cambium suggests that it participates in the complex of reactions which result in lignification. However, its occurrence in parts which are not lignifying indicates that this cannot be its sole function.

Other enzymes have been shown to be particularly active in the cambium of some plants, e.g. peroxidase and other glycosidases (van Fleet, 1950; Freudenberg, Reznik, Boesenberg and Rasenack, 1952). In the latter work a histochemical method identical in principle with the galiosinase method was employed. Sections of plant tissues were treated with a solution of indican (indoxyl-β-glucoside) under conditions of aeration. The cells containing β-glucosidase stained blue owing to the deposition of indigo blue, which is very insoluble and is formed by the oxidative polymerization of indoxyl released from indican by the action of the glucosidase. Shoots of the Norfolk Island pine, *Araucaria excelsa*, showed the localization of β-glucosidase on the inner side of the cambium and the outer part of the woody zone, which is not fully lignified. Other histochemical techniques showed the presence of phenol dehydrogenase on either side of the cambium. *In vitro*, phenol dehydrogenase brings about the formation of lignin-like substances by the oxidative polymerization of compounds of the *p*-hydroxycinnamyl alcohol type, e.g. coniferyl and syringyl alcohol. β-glucosidase activity was also found to be localized in the lignifying zone of *Cordyline congesta* (Liliaceae). On the basis of these observations and experiments with *in vitro* models Freudenberg *et al.* postulate the formation of lignin in the living cell by a process of hydrolysis of β-glucosides of the *p*-hydroxycinnamyl alcohol type, localized in the region of lignification, followed by enzyme catalysed oxidative polymerization of the phenolic substances thus released.

The evidence for the course of lignification process proposed by Freudenberg *et al.* is not conclusive. The present observations on the distribution of galiosinase, which also show a very close association of a glycosidase (galiosinase) with lignifying cells, do not appear to confirm Freudenberg's hypothesis but provide grounds for questioning it. For the substrates upon which galiosinase acts are not very closely related in structure to lignin. However, great structural changes in the anthraquinone molecule might be effected by enzyme action and it might be finally converted to lignin. Alternatively, since the xylem is not entirely composed of lignin, the anthraquinones may be converted to other kinds of xylem deposits.

Offering as they do great difficulties in interpretation, the above results *do* show that glycosides and glycosidases must be involved in the metabolic activity of meristematic tissues and act particularly as a stimulus to the investigation of the role of glycosides and glycosidases in the lignification process.

The formation of large quantities of galiosinase in excised tissues, which when fresh contain none, is an interesting phenomenon and cannot be dismissed as a consequence of autolysis in disorganized cells, since it is invariably followed by the appearance and accumulation of purpurin-3-carboxylic acid glucoside and galiosin in the same cells. The enzyme must arise either from an inactive zymogen form or by synthesis *de novo*.

There are many recorded instances of enzymes in a bound zymogen form in plants. Thus invertase in the root of the sugar-beet exists almost entirely in a bound and apparently inactive form, from which it is released by autolysis, as was first shown by Stoklasa (1903). Other hydrolytic enzymes have been found to exist partly or entirely in a zymogen form in many plants. On the other hand, *de novo* synthesis of enzymes has not been shown to occur in fully developed plant tissues and it would seem to be unlikely to occur in starved, excised tissues which normally undergo strong protein breakdown. However, the synthesis of protein, including enzymes, must presumably occur where there is tissue regeneration, a common occurrence in excised plant tissues.

This discussion leads naturally to a consideration of the role of particular enzymes and their substrates in the differentiation and regeneration of plant tissues. In Stellateae the anthraquinone pigments are essentially products of the root and it is only purpurin-3-carboxylic acid glucoside and galiosin which will accumulate in detectable quantities in other parts of the plant, always associated with galiosinase. It appears significant that adventitious rootlets arise most readily from those aerial parts of the plant which show the greatest tendency to produce galiosinase and purpurin-3-carboxylic acid glycosides, namely the nodes and the base of the shoots. Consequently it may be profitable to investigate the behaviour of excised parts of rubiaceous plants working on the following hypothesis: these excised parts are in the process of regeneration, the first stage of root formation being marked by the formation of galiosinase followed by the accumulation of the purpurin-3-carboxylic acid glycosides, and later, after root cells begin to develop, by ruberythric acid and its specific hydrolytic enzyme erythrozyme and the other anthraquinone glycosides, such as rubiadin primveroside, all of which seem to occur in the fully formed root.

The complexity of the patterns of enzyme and substrate distribution described in this paper is emphasized by the fact that in the Indian madder, *Rubia cordifolia*, the green parts of the plant contain amounts of purpurin-3-carboxylic acid glucoside in sufficient quantities to permit chemical isolation of the pure crystalline glucoside (Hill and Trim, unpublished). It has not been possible to obtain further supplies of this plant in order to study the quantitative distribution of galiosinase in the shoots, but experience in the isolation procedure (Hill, private communication) showed that the enzyme must be present in considerable quantities, thus distinguishing *R. cordifolia* from the plants described in this paper.

The system which has been studied is of a highly specialized character and may be confined to Stellateae, but there are large numbers of more or less specific hydrolase-substrate systems in all living organisms and as is so readily shown in this special case they must play an important part in the integration and differentiation of tissues. This study based on simple, rapid methods has permitted a large number of observations on numerous closely related plants. Certain generalizations begin to emerge and show the way for the development of work on these lines, perhaps with less specialized enzyme systems, with the object of establishing a firmer bridge between enzymological and chemical studies and the many phenomena of plant physiology which must be based on cellular activities but which so far have yielded poorly to biochemical investigation.

ACKNOWLEDGEMENTS

The author is indebted to Dr. R. Hill, F.R.S., for making available plant material from his collection of Rubiaceae, to Mr. E. T. Whitmore for technical assistance in part of the work, Mr. Edward Leigh, F.I.B.P., F.R.P.S., for photographs, and to the Agricultural Research Council for financial support.

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Observations with the Electron Microscope on Biciliate and Quadriciliate Zoospores in Green Algae

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WITH 4 PLATES

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SUMMARY

Quadriciliate zoospores of *Draparnaldia* and biciliate zoospores of *Chaetomorpha* have been examined using our previous methods. New features have been seen in both, chiefly in connexion with the basal organs which attach the cilia to the cytoplasm of the parent cell.

MUCH is now known about ciliary structure in plants, although the green algae themselves have hitherto been rather scantily treated. It is true that in our own previous papers they have been mentioned three times, viz. Manton (1952) with two micrographs of *Ulothrix* and a preliminary statement on *Draparnaldia*, and Manton (1953) with one micrograph of *Chaetomorpha*, but these are all concerned with one detail only, namely, the demonstration of the 11-stranded structure. There are many other facts, however, which can be obtained from this material and which are of interest for comparative purposes. With some of these we now propose to deal, using the same species of *Draparnaldia* and *Chaetomorpha* to which preliminary reference has already been made.

Draparnaldia, as is well known, is a common freshwater alga which liberates quadriciliate zoospores profusely in the late spring. One of these is shown intact in Pl. I, Fig. 1, and the tips of its rather blunt cilia appear in Pl. I, Fig. 2. These cilia dismember very freely, and had the 11 strands been still in doubt we could have demonstrated them with great clarity. Many cilia also split longitudinally along a single line, thereby revealing more clearly than is usual the tubular nature of the ring of 9 strands and their freedom from the central pair which are indeed in this material liable to fall out. These details are shown in Pl. I, Fig. 4.

Another characteristic feature, which we have encountered also in the quadriciliate zoospores of *Ulothrix*, is that the whole cell may break up in such a way as to cause the tuft of 4 cilia, together with all their basal parts, to be pulled out of the cytoplasm either relatively undamaged or else damaged in such a way by dismemberment that internal structure in the basal parts is revealed.

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Two electron micrographs in illustration of this are reproduced. Pl. II, Fig. 5, shows a tuft of 4 cilia each dismembered into its 11 strands. Diagonal cross-markings are still visible which we interpret as remains of the internal spiral winding, detected previously in *Sphagnum* and *Pylaiella* (Manton and Clarke, 1952), which in life holds the tube of 9 strands together. At their bases the cilia pass abruptly into denser bodies of obviously different structure which recall the 'bulbous bases' previously noticed in the fungi *Allomyces* and *Olpidium* (Manton, Clarke, Greenwood, and Flint, 1952). Between these basal bodies emerge fibrous 'roots' which are clearly of different sizes, two of them having 2 component strands and the other two 5 component strands.

Some indication of the position of the fibrous 'roots' in the intact cell can be obtained from Pl. I, Fig. 3, and we have exactly comparable evidence in *Ulothrix*. In both, the path of some, though perhaps not all, of the 'roots' lies immediately under the surface of the cell passing backwards from the point of insertion of the cilia. Where the roots end cannot be seen.

Another type of view of the basal apparatus of the 4 cilia is seen in Pl. III, Fig. 6. The cilia have here become broken away, although 2 are still almost in position and the basal bodies of all 4 are clearly discernible. They have, however, become somewhat pulled apart and the fibrillar structure of 2 of the 'roots' is visible on the right-hand side of the specimen although those on the left are intact.

We are not yet in a position to interpret every detail shown by Pl. III, Fig. 6, but we have seen enough duplicate specimens of this kind to say with reasonable certainty (*a*) that the basal bodies of the cilia are arranged in a ring in which they are joined to each other by fibrous connexions; (*b*) that the 'roots' arise between the basal bodies and not directly from them although each root has fibrous connexions with both of the adjacent basal bodies; and (*c*) that the rather extreme length of 'roots' after dismemberment is an artefact (they are sometimes longer than the whole length of the intact cell), but there is probably a genuine difference of length between them, the 5-stranded roots being short and apparently ending abruptly while the 2-stranded roots are perhaps attached to some other cell organ (cf. right-hand lower root in Pl. III, Fig. 6).

When we turn to the biciliate zoospores of *Chaetomorpha*[1] the basal portions are surprisingly similar in spite of the smaller number of cilia to which they relate. One such zoospore is shown in Pl. IV, Fig. 7, with the tip of one of its whiplash cilia in Pl. IV, Fig. 8. The basal portions are shown in Pl. IV, Fig. 10, with a dismembered cilium treated in the same manner in Pl. IV, Fig. 9. The last two specimens are stained and stripped from glass (cf. Manton,

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[1] Plants of this seaweed were collected near Cullercoats in January 1952 by Dr. E. Nicolai, who identified them as *C. melagonium*. On being put into fresh sea-water in the laboratory in Leeds, they liberated zoospores copiously. Preparations were made both on glass slides and on carriers by our ordinary methods. All our observations relate to this one gathering which proved to be more informative than those of several other marine green algae which we have attempted to study. We are very grateful to Dr. Nicolai for this timely gift.

Clarke, and Greenwood, 1953), since dismemberments in this species are too infrequent to be found without the aid of visual microscopy.

As is shown by Pl. IV, Fig. 10, there are still four 'roots' in spite of the fact that there are here only two basal bodies to correspond to the smaller number of cilia. The staining has obscured all details within the basal bodies and also the details of origin of the roots. Their fibrillar composition is, however, revealed and we have several duplicate specimens showing the same structure. As before there are two sizes: two of the roots have 2 component strands and the other two have 3. The latter presumably correspond to the 5-stranded roots in the quadriciliate zoospores.

This is as far as our present analysis has reached, though we hope shortly to examine these structures further by other methods. In the meantime it is perhaps worth commenting on the striking difference which undoubtedly exists between the rigid uniformity[1] of internal structure among all known ciliary types and the diversity which is at once revealed when we examine their ancillary appendages. The tips of the cilia in *Draparnaldia* and *Chaetomorpha* are more unlike than their phyletic positions might lead one to expect, from which one may perhaps conclude that here we have a relatively trivial variable which will not tell us much. The basal structures, however, seem to show just that type of variation which is in part functional, in this case connected with a difference in the number of cilia, but in part may be of phyletic significance. The two genera show a strong family likeness in just those characters in which they differ from, for example, the brown algae in which comparable parts have been investigated in *Himanthalia* and *Dictyota*. Further insight into the micro-anatomy of the organs internal to the cell is therefore greatly to be desired.

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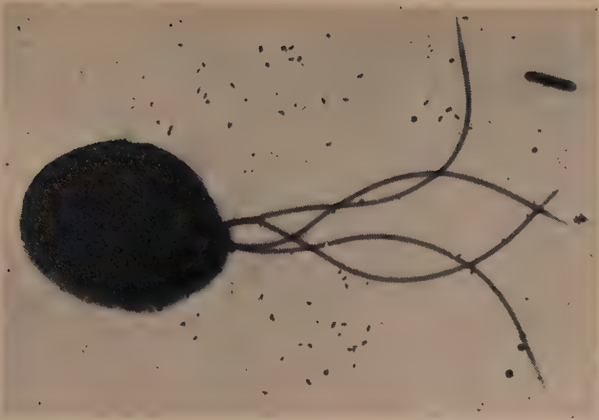
[1] We do not regard Lewin's failure regularly to detect 11 strands in the cilia of *Chlamydomonas* as constituting an exception (Lewin and Meinhart, 1953), since this otherwise excellent piece of work involved preliminary treatment of the cilia which is unlikely to have prevented loss of the central strands, which can often occur very rapidly (cf. our own similar experience in *Fucus* discussed in Manton, Clarke, and Greenwood, 1953).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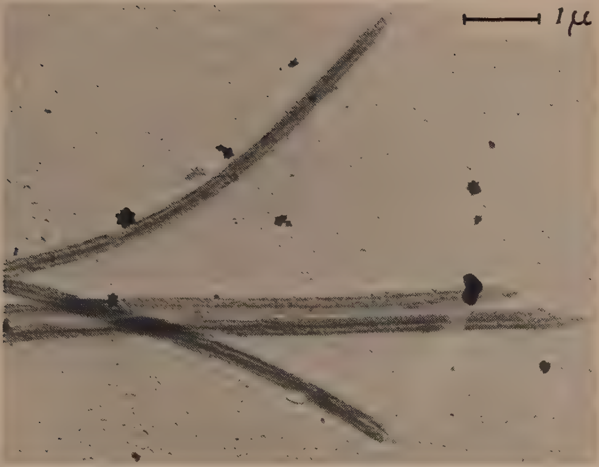

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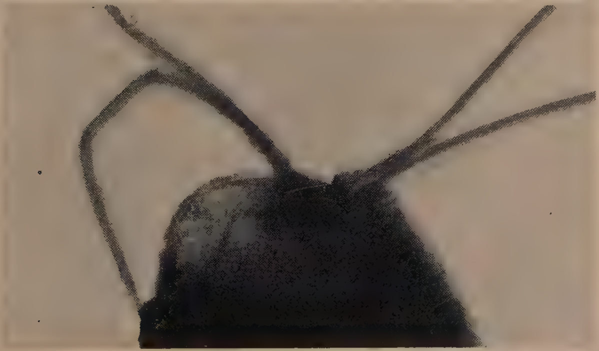
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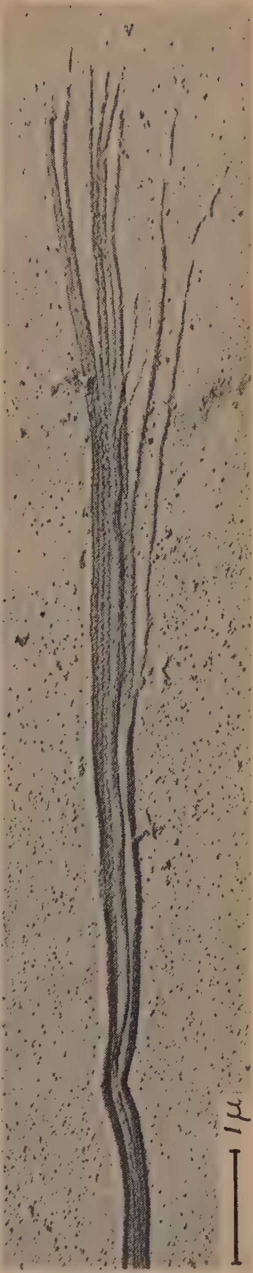

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1. *Draparnaldia* sp., a quadriciliate zoospore. Electron micrograph M80.12, gold-palladium shadowing, 60 kV; magnification × 3,000.

2. Tips of the four cilia from another cell of the same. Electron micrograph M82.30, 60 kV; magnification × 10,000.

3. Bases of the four cilia from another cell of the same showing traces of the 'roots' in position. Electron micrograph M90.22, 80 kV; magnification × *c.* 7,000.

4. A cilium split down one side. Electron micrograph M81.2, gold-palladium shadowing, 60 kV; magnification × 15,000.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE I

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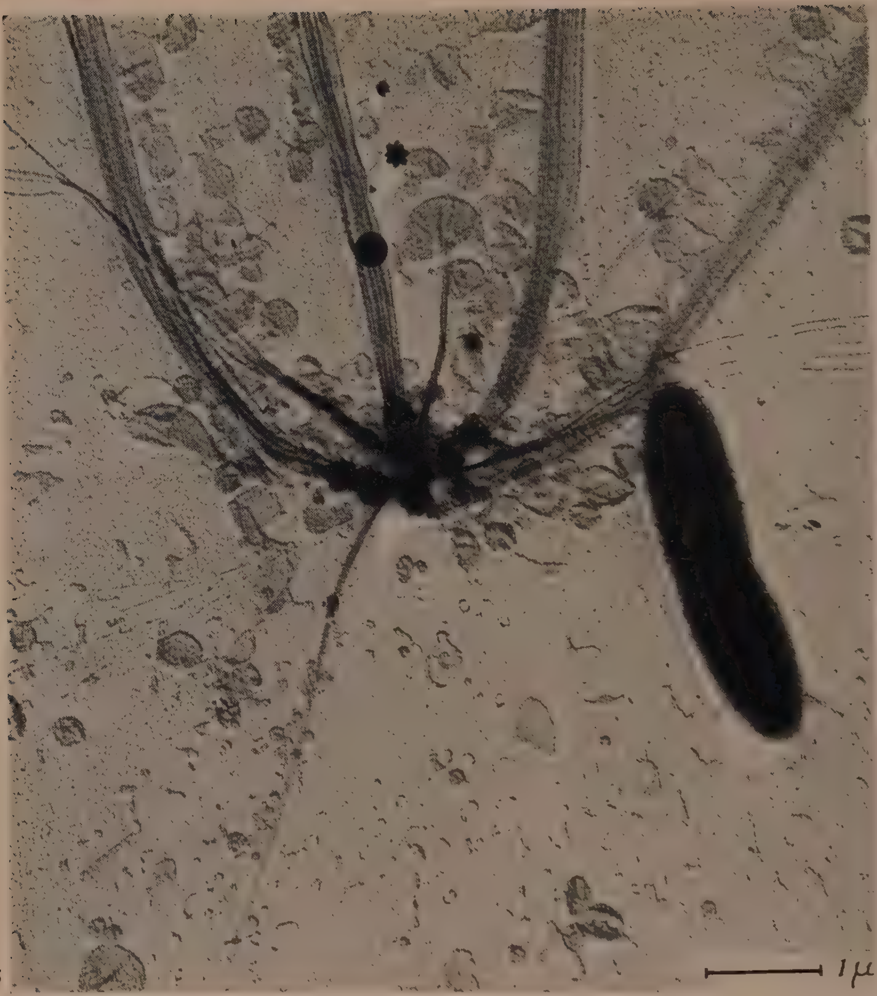

5. *Draparnaldia*, the basal parts of four cilia of a disrupted zoospore. Electron micrograph M82.22, gold-palladium shadowing, 60 kV; magnification × 15,000.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE II

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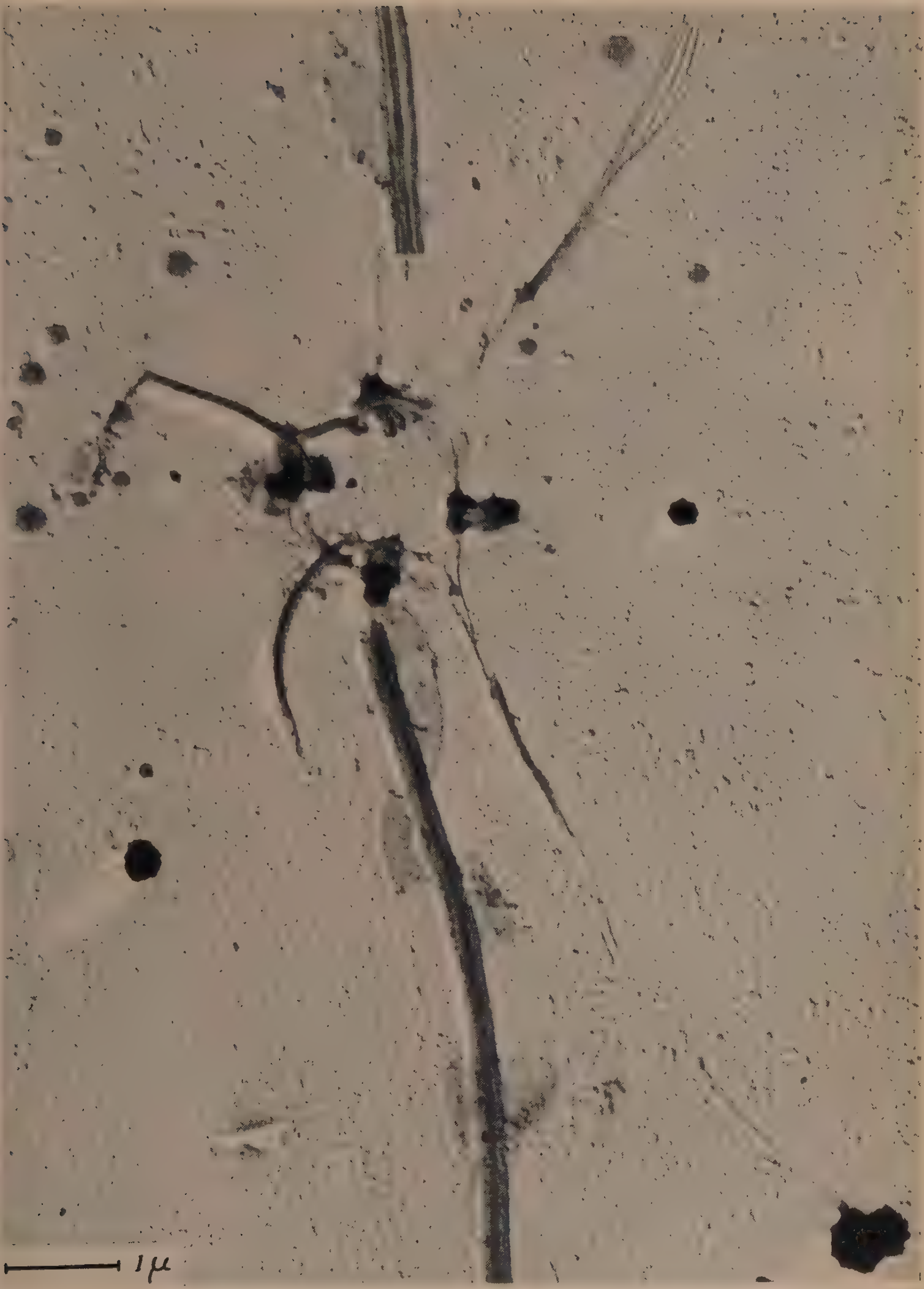
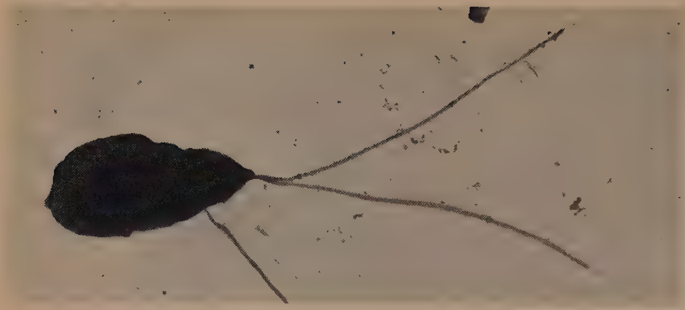


6. *Draparnaldia*, polar view of the bases and roots from a tuft of four cilia from a disrupted zoospore. Electron micrograph M89.25, gold-palladium shadowing, 60 kV; magnification × 15,000.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE III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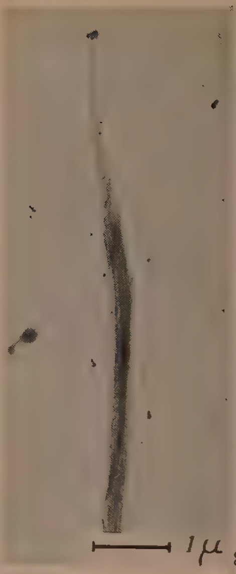

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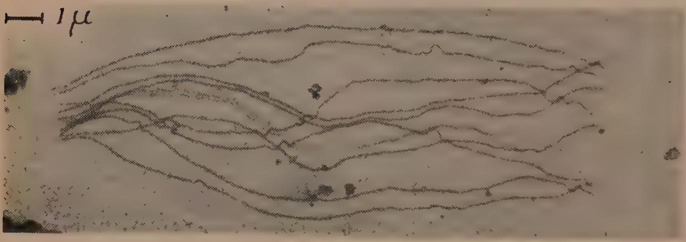

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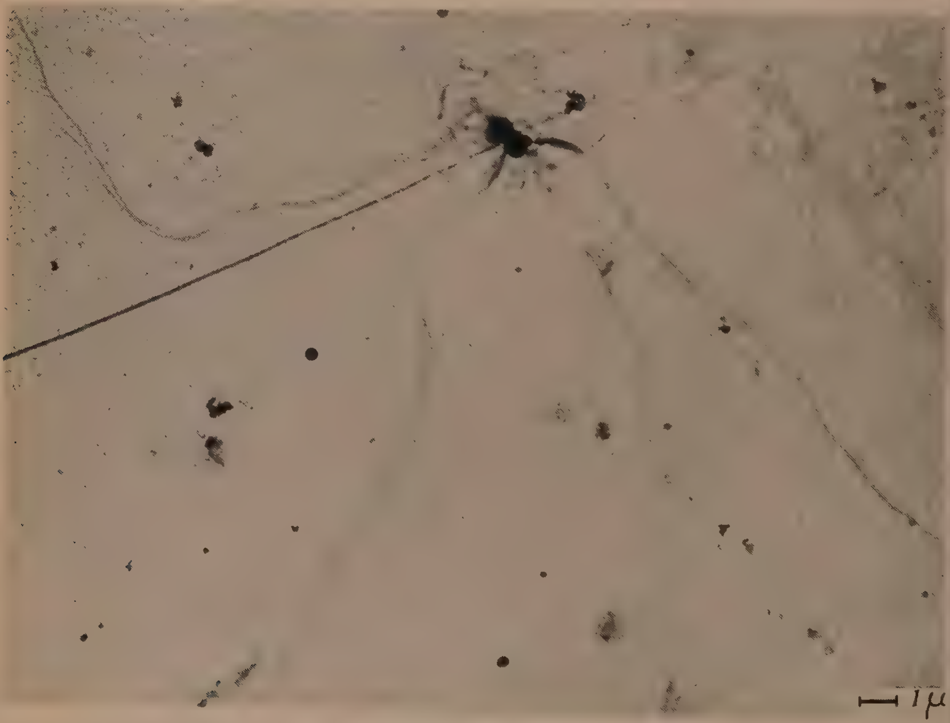

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7. *Chaetomorpha melagonium*, a biciliate zoospore. Electron micrograph M83.22, gold-palladium shadowing, 80 kV; magnification × 3,000.

8. Tip of an intact cilium for comparison with *Draparnaldia*. Electron micrograph M83.23, gold-palladium shadowing, 80 kV; magnification × 10,000.

9. Detached cilium showing fibrillar construction, stained and stripped from glass. Electron micrograph M102.22 60 kV; magnification × 5,000.

10. Polar view of bases and roots from a disrupted cell otherwise like the preceding. Electron micrograph M 102.3, 60 kV; magnification × 5,000.

I. MANTON, B. CLARKE, A. D. GREENWOOD—PLATE IV

The Growth Substances separated from Plant Extracts by Chromatography. I[1]

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SUMMARY

Methods for the chromatographic separation on paper of indole compounds and for the direct biological assay of the chromatograms using the *Avena* coleoptile straight-growth method are described. Reagents for the detection of the indole-3-carboxylic acids, indole-3-acetonitrile, and gramine as coloured spots on chromatograms are compared and the areas of such spots are shown to be proportional to the logarithms of the quantities of substance present.

The procedure of chromatography described is shown not to involve a loss of indole-3-acetic acid activity if chromatography is done in darkness and chromatograms are not stored in light and air.

Methods are described for the extraction of growth substances from plant materials, the purification and chromatography, on paper, of the extracts and the bioassay of the chromatograms using *Avena* coleoptile sections.

The ether extracts, containing acidic substances, of etiolated broad bean and pea shoots and roots, etiolated sunflower shoots, maize roots, and potato etiolated shoots and tuber have been chromatographed and the chromatograms bioassayed. On all chromatograms three areas active in *Avena* coleoptile section growth are found. One area of growth promotion is shown due to indole-3-acetic acid [IAA]. Another area of growth promotion and one of growth inhibition are due to unknown substances, which are named accelerator α (α) and inhibitor β (β) respectively.

On chromatograms of potato tuber a fourth growth-promoting area, in addition to those described above, is detected and is shown to be probably due to indole-3-acetonitrile [IAN]. IAN or indole-3-pyruvic acid may occur together with IAA on chromatograms of extracts of immature maize kernels and cauliflower head respectively.

On cabbage extract chromatograms the growth-promoting activity corresponding in position with IAA is shown to be due to IAA and to IAA alone.

In etiolated broad bean shoots IAA is the predominating growth substance in the stem and β predominates in the first lateral bud. The latter is suggested as an explanation of apical dominance, and the predominance of β in potato tuber skin is suggested as an explanation of dormancy in tubers.

In the broad bean root the acidic growth-substance patterns, for the whole root and for the sections 0–2 cm. and 2–4 cm. from the tip, are the same.

The acidic growth substances extractable from broad bean shoots are the same whether the plant material is boiled or frozen before extraction.

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[1] This work formed part of a thesis presented to the University of London.

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INTRODUCTION

To explain the results of their study of the geotropic behaviour of the rhizomes of *Aegopodium podagraria*, Bennet-Clark and Ball (1951) had to postulate the presence in the rhizomes of at least two growth substances with different rates of movement under the influence of gravity. These authors pointed out that this theory, which could be applied to other types of geotropic behaviour, was best tested experimentally by the separation and assay of growth substances in plants and it was with this end in view that an investigation of the chromatographic separation and assay on paper, of the growth substances in plant extracts, was initiated in this laboratory. Clearly this was not the only end to be served by the development of a separation technique, as has been shown by the fact that, while the present work has progressed, other workers, mentioned below, have adapted the technique and selected the study material to suit their particular purposes. The importance of proper identification of growth substances is emphasized in recent reviews.

Some growth substances have been separated from plant extracts by Bien *et al.* (1947) and Linser (1951) using adsorption column chromatography and by Holley *et al.* (1951) using the counter-current partition separation method. Neither of these techniques is so versatile or so simply adaptable to small amounts of material as is partition chromatography on paper.

In this laboratory Tambiah (1951) investigated the chromatography of indole-carboxylic acids and did much preliminary work particularly in the choice of chromatographic solvent, which is mainly empirical. He investigated the suitability of a number of solvents, using as his criteria a low R_F value for indole-3-acetic acid (IAA), chemical non-reactivity between solvent and the indole-carboxylic acids, complete evaporation of solvent from paper on drying, and separation on chromatograms of spots of IAA, indole-3-propionic acid (IPA), and indole-3-butyric acid (IBA). Tambiah (Tambiah, 1951; Bennet-Clark, Tambiah, and Kefford, 1952) found that a mixture of *iso*propyl alcohol:water:ammonia ($D = 0{\cdot}880$)::10:1:1 was the most useful solvent and this, or mixtures with *n*-butyl alcohol, have been used by Luckwill (1952), Pacheco (1951), Jerchel and Müller (1951), von Denffer and Fischer (1952), Terpstra (1953), Lexander (1953), and Vlitos and Meudt (1953). After a systematic study of a wide variety of chromatographic solvents and involving over thirty indole compounds, Stowe and Thimann (1953) concluded that an *iso*propanol-water-ammonia mixture, similar to that of Tambiah was best for the separation on paper of the indole-carboxylic acids. Yamaki and Nakamura (1952) found that for their purpose 70 per cent. ethanol, phenol saturated with water, and ligroin saturated with water each gave a clear-cut spot of IAA on chromatograms, and for indole-3-acetaldehyde (IAc) 70 per cent. ethanol was used.

To detect spots of indole-carboxylic acids on developed chromatograms, Tambiah (1951) used a mixture of sodium nitrite solution and nitric acid, a Salkowski reagent, which had been used by Mitchell and Brunstetter (1939) for the quantitative estimation of IAA. Ferric-chloride and the acids, sulphuric (Tang and Bonner, 1947), hydrochloric, and perchloric (Gordon and Weber, 1951), have been used as reagents producing colour reactions with IAA and these have been adapted for spraying chromatograms (Bennet-Clark *et al.*, 1952; Yamaki and Nakamura, 1952; Stowe and Thimann, 1953). Terpstra (1953) used a spray of cinnamic aldehyde in methanol in the presence of hydrochloric acid gas, and Vlitos and Meudt (1953) used a solution of *p*-dimethylaminobenzaldehyde to detect IAA. Spots of IAc have been detected with a spray of benzidine in acetic acid (Yamaki and Nakamura, 1952) and indole-3-acetonitrile (IAN) and indole-3-aldehyde with mixtures of sodium nitrite and hydrochloric acid (von Denffer and Fischer, 1951; von Denffer *et al.*, 1952) and sodium nitrite and nitric acid (Bennet-Clark and Kefford, 1953).

A method for the direct quantitative biological assay of growth substances on paper cut from chromatograms has been developed (Tambiah, 1951; Bennet-Clark *et al.*, 1952) using the *Avena* coleoptile section extension test and Luckwill (1952) used a modification of this method.

The experiments recorded here have been reported in part in two preliminary notes (Bennet-Clark *et al.*, 1952; Bennet-Clark and Kefford, 1953).

The chemical structure of some naturally-occurring plant growth substances are known with certainty. Recently it has become increasingly evident that indole-3-acetic acid (IAA) is a major natural auxin in higher plants (Bonner and Bandurski, 1952). Indole-3-acetonitrile (IAN) was isolated from cabbage and shown to be an auxin by Jones *et al.* (1952), and its other properties were investigated by Bentley and Housley (1952; 1953), Bentley and Bickle (1952), Osborne (1952), and Thimann (1953). The latter presented evidence for IAN having its auxin action through a conversion to IAA. Indole-3-pyruvic acid (IPyA) has been found in corn seed (Stowe and Thimann, 1953) and it is reasonably well established that indole-3-acetaldehyde (IAc) occurs in plants. The properties of the latter have been reviewed by Larsen (1951) and added to by Yamaki and Nakamura (1952) and Bentley and Housley (1952). There is considerable doubt about the occurrence of auxin *a* and *b* in plants, and this doubt is due to the lack of direct confirmation of the original work on urine, maize oil, and malt, and to the methods giving indirect evidence of the occurrence of these substances, namely molecular weight by diffusion and acid-alkali stability having been shown to be unreliable (Bonner and Bandurski, 1952; Terpstra, 1953). Bennet-Clark (unpublished report), who suggested that the urine from which auxin *a* was obtained may have been pathological and thus not reproducible, examined a pathological urine with extraordinarily high plant-growth activity, but found chromatographically that all of the activity was due to IAA, which was present at about 200 mg./l. Some workers (Jones *et al.*, 1952; Terpstra, 1953; Söding and Raadts, 1953; Bennet-Clark *et al.*, 1952) have sought auxin *a* or *b* in plant material or urine without success, which was the case in the present investigation. Doubt has been cast upon the chemical properties that have been claimed for auxin *b*. Brown *et al.* (1950) prepared a compound with the same ring system and major side chain as auxin *b* and found that it could exist only as a lactone under all conditions. This and a thorough investigation of the derivatives claimed for auxin *b* cast doubt on the structure proposed for it. In addition a number of analogues of auxin *b* lactone were found inactive in the *Avena* coleoptile section test and the cress root test.

The extraction of growth substances from plant materials has been the subject of controversy for some time. One great difficulty is that the chemical structures of some of the growth-active substances occurring in plants is not known and those that are known have only recently had their structures established with certainty. This has meant that, in the past, estimates of the efficiency of extractions have been based on the rather vague determinations of general growth activity of extracts or diffusates; however, chromatography on paper now offers a means of separating the general growth activity of extracts into components and of quantitative estimation of the known substances. Although large-scale sources of unknown growth substances have been sought, the main emphasis of this investigation has been physiological. Thus seedlings were mainly used in the preparation of extracts because of their possible use in future growth experiments.

A number of authors have given support to a theory of the mechanism of a plant function with an assay of the general growth activities of unpurified extracts or diffusates from plant tissue. One instance of this is the support given to the Cholodny–Went theory of plant responses by such assays of the growth activities of extracts or diffusates from the two sides of a stimulated organ (reviewed by van Overbeek, 1939 and 1944; Went, 1939; Shrank, 1951). Before support of any theory can be claimed, this type of experiment must be repeated with purified extracts and with some attempt to separate and assay the various growth substances that may be present in the extracts.

Growth substances are known to be formed and inactivated by enzymes occurring in the tissues from which they are extracted, and unless precautions are taken, these processes can occur during extraction. Hence the first concern in the preparation of an extract of a plant material which is to contain all of the growth substances present on harvesting, and only these, is to inactivate the enzymes. Boiling of the tissue is an obvious method of enzyme inactivation, but may sometimes be objectionable (cf. van Overbeek *et al.*, 1945; Terpstra, 1953). The use of enzyme inhibitors for inactivation was ruled out by Wildman and Muir (1949) because they found potassium cyanide to interfere with subsequent biological testing, but Terpstra (1953) found that sodium diethyl-dithiocarbamate, which inhibited the IAA-destroying enzyme in *Avena* coleoptile tips, did not interfere with the *Avena* curvature test. Gustafson (1940) suggested an extraction of dried plant material with dry ether, under which conditions enzymes would be inactive, but when Link *et al.* (1941) and Wildman and Muir (1949) applied this method to a number of materials they were unable to extract any growth-active substances. A quick extraction of relatively intact tissue with ether at room temperature was advocated by van Overbeek *et al.* (1945, 1947) for the determination of 'free' auxin, with a longer period of extraction to determine the auxin due to the enzymatic breakdown of 'bound' auxin; however, the change over from 'free' to 'bound' was not distinct. Wildman and Muir (1949) recommend extraction with ether for 16 hours at near 0° C., but Terpstra (1953) found that auxin formation in *Avena* coleoptile tips was not stopped in ether at 4° C. and at this temperature a neutral growth substance accumulated. Thimann and Skoog (1940) found that with absolute alcohol as solvent the continuous extraction of growth substances from plant material over a long period, which occurs in wet ether or water, did not take place, presumably due to the dehydrating effect of the alcohol on the enzymes. The extraction method employed in the present work made use of the enzyme inactivating properties of absolute alcohol as solvent and of the temporary enzyme inactivation obtained by freezing and extraction at −10° C.

In the literature (reviewed by Bonner, 1950; Haagen-Smit, 1951; Larsen, 1951) there is much discussion on auxin-protein complexes, other forms of bound auxin and auxin precursors, and these should be considered in a picture of the growth-substance status of a plant tissue.

Early in the study of extracts of auxins from plant materials, ether became the common solvent. This proved an unfortunate choice since, in ether, plant tissues produce auxin enzymatically for a long period and, for some years, this was wrongly interpreted as proving that ether was the most efficient solvent for complete auxin extraction. Tambiah (1951) found that after extracting a *boiled* pulp of sunflower seedlings at pH 2 three times with ether, no more IAA could be extracted by further ether extractions and that synthetic IAA added to the pulp could be re-extracted in 90 per cent. yield.

Conditions for the chromatographic separation of acidic growth substances in plant extracts, similar to those worked out by Tambiah (Tambiah, 1951; Bennet-Clark *et al.*, 1952), have now been used by a number of authors (Jerchel and Müller, 1951; Luckwill, 1952; von Denffer *et al.*, 1952; Yamaki and Nakamura, 1952; Hancock and Barlow, 1953; Terpstra, 1953; Lexander, 1953; Stowe and Thimann, 1953; Vlitos and Meudt, 1953; Wolf, 1952; Teubner, 1953; Müller, 1953; Bolle-Jones, 1954; Weller *et al.*, 1954).

METHODS

Chromatography of indole compounds. Solutions of synthetic growth substances were made in absolute alcohol and were stored in darkness at −10° C. Sheets of Whatman No. 1 chromatography paper were used and the substances were applied, to the starting lines of chromatograms, as alcoholic solutions from a calibrated micropipette of 3 μl. volume. They could be applied to the starting line as solutions in ether from a dropping pipette as is used for the application of plant extracts. On all chromatograms destined for bioassay, marker spots of IAA or IAN were applied at one end of the starting line so that a strip could be removed, after development, for spraying.

Chromatograms were allowed to equilibrate, with an atmosphere saturated with chromatographic solvent, for 8 to 16 hours and were developed, with solvent descending, for times ranging from 4 to 16 hours at a constant temperature of 27° C. After development, chromatograms were dried in a draught, out of direct sunlight, and were immediately sprayed or prepared for bioassay. In the major part of this work *iso*propanol:water::10:1 was the running solvent and *iso*propanol:water:ammonia ($D = 0{\cdot}880$)::10:1:1 was the equilibrating solvent.

For the detection of spots of indole compounds, chromatograms were sprayed with one of the following mixtures from an atomizer. The titles given here to the spray mixtures will be used throughout in the text.

1. Ferric-perchloric. 5 per cent. perchloric acid:0·05 M. ferric chloride::50:1.
2. Nitrite-nitric. Concentrated nitric acid:0·5 per cent. sodium nitrite::1:1.
3. Nitrite-perchloric. 5 per cent. perchloric acid:0·5 per cent. sodium nitrite::1:1.
4. Ferricyanide-nitric. Concentrated nitric acid:0·5 per cent. sodium nitrite::1:1.
5. Ferric-sulphuric. 40 per cent. sulphuric acid:0·5 M. ferric chloride::50:1.
6. Ehrlich's reagent. 0·5 g. dimethyl-*p*-aminobenzaldehyde and 1 ml. concentrated hydrochloric acid in 100 ml. ethyl alcohol. A more satisfactory spray contains 50 per cent. (v/v) of HCl rather than 1 per cent.

When full colour was attained the limits of the spots were marked and the areas were measured with a planimeter; areas are recorded here in planimeter units.

Biological assay of chromatograms with the Avena coleoptile section Extension test. Husked *Avena sativa* (var. 'Victory' from Svalof) seeds were grown in a standard manner as given by Bennet-Clark and Kefford (1954). Straight uniform coleoptiles were placed, 12 at a time, in a cutter which took a section approximately 3 mm. long after removing a 3 mm. tip.

Chromatograms to be bioassayed were cut into pieces 2·5 cm. long and 2·5 cm. or more broad and, where possible, one square was made to correspond with the position of a sprayed marker spot. The paper pieces were placed in pyrex dishes 3·5 cm. diameter and blank paper and paper holding known amounts of IAA or IAN were included as control and standards. To every test dish 2 ml. 3 per cent. sucrose (AR) in glass-distilled water was added, then an average of 10 coleoptile sections were floated on the surface of the solution and the whole was gently agitated for 20 hours, when the length extension of the sections was measured, using a microscope fitted with a micrometer eyepiece. In the tables and figures the elongation of the coleoptile sections has been expressed as the percentage extension over the original length and the standard errors of these percentages are given.

Colour of spots of indole compounds on spraying. As a number of colour reagents for indole compounds were known, the ones most satisfactory for the detection of spots of indole-carboxylic acids, IAN and gramine (GRM), were sought. In Table I are shown results of a number of experiments in which spots of 1 μg. of IAA, indole-3-propionic acid (IPA) and indole-3-butyric acid (IBA), 3 μg. of IAN and 10 μg. of gramine were chromatographed, then the dried papers were immediately sprayed and colours and intensities studied. The paper sprayed with ferric-sulphuric was the only one that did not dry. The spots of the indole-carboxylic acids, sprayed with nitrite-nitric, ferricyanide-nitric, and nitrite-perchloric, had edges that were difficult to discern. With IAN and gramine some sprays gave no colour. It appeared that, of the sprays tried, ferric-perchloric was most satisfactory for the indole-carboxylic acids and nitrite-nitric for IAN and gramine. This point receives further support from area measurements.

TABLE I

The colour and intensity of colour of spots of indole compounds on chromatograms developed in aqueous isopropanol and sprayed with various reagents

Compd.	Spray	Colour	Intensity
IAA	ferric-perchloric	crimson	strong
	ferric-sulphuric	crimson	strong
	nitrite-nitric	pink	strong
	nitrite-perchloric	pink	strong
	ferricyanide-nitric	crimson	strong
	Ehrlich's + 1% HCl	blue	weak
	,, + 50% HCl	blue	strong
IPA	ferric-perchloric	buff	medium
	nitrite-nitric	orange	weak
	nitrite-perchloric	orange	weak
	ferricyanide-nitric	buff	weak
IBA	ferric-perchloric	orange	medium
	nitrite-nitric	yellow	weak
	nitrite-perchloric	yellow	weak
	ferricyanide-nitric	fawn	weak
	Ehrlich's	blue	weak
IAN	ferric-perchloric	none	—
	nitrite-nitric	blue-green	strong
	Ehrlich's + 1% HCl	none	—
	,, + 50% HCl	greenish brown	strong
GRM	ferric-perchloric	none	—
	nitrite-nitric	green	medium
	Ehrlich's + 1% HCl	none	—

The spot area—logarithm quantity relationship for indole compounds. The results of an experiment in which chromatograms of series of amounts of IAA were sprayed with ferric-perchloric, nitrite-nitric, or ferricyanide-nitric sprays and spot areas measured are plotted in Fig. 1 as spot areas against logarithms of quantity. Although the chromatograms were not strictly comparable, since they were developed on different sheets of paper, it would appear that ferric-perchloric is a more sensitive spray detector than ferricyanide-nitric or nitrite-nitric spray. Spot areas for small quantities of IAA and series of quantities of IPA and IBA on chromatograms sprayed with ferric-perchloric have been plotted against logarithms of quantity in Figs. 2*a* and 2*c*. It was clear that a linear relationship between spot area and logarithm of quantity held for IAA over the range 0·25 to 8·0 μg. and for IPA and IBA over the range 2·0 to 20 μg., when the spots were applied with a micropipette and the chromatograms sprayed with ferric-perchloric reagent. The linearity also held when IAA was applied to a chromatogram from a dropping pipette. It should be mentioned that with the ferric-perchloric spray smaller quantities of the indole-carboxylic acids could be detected qualitatively than were stated as the lower limits for area measurement. A linear relationship between the area and logarithm of quantity was also found to hold for spots of IAN and gramine on chromatograms sprayed with nitrite-nitric spray.

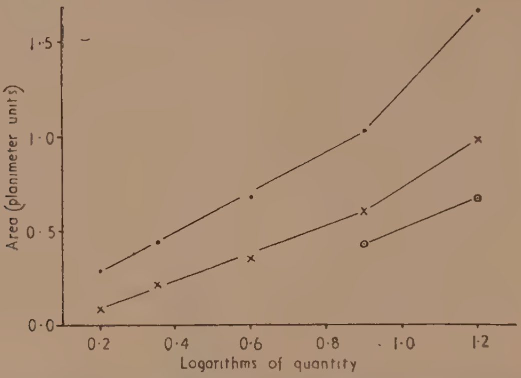

FIG. 1. The relationship between area and logarithm of quantity for spots of IAA on chromatograms sprayed with ferric-perchloric (—●—), ferricyanide-nitric (—×—), or nitrite-nitric (—o—) reagents.

The loss of IAA activity on chromatograms. Tambiah (1951), who often stored chromatograms overnight before equilibration and development and always allowed them to dry in air overnight before spraying or bioassay, found a loss of about 60 per cent. of IAA on the chromatograms, which was overcome by running the chromatograms in an atmosphere of nitrogen. This important point was investigated further by preparing three chromatograms, two of which were developed in air and the third was developed in a tank that had been filled with nitrogen. The latter and one of those run in air were sprayed with ferric-perchloric spray immediately after drying. The areas of the spots are plotted against the logarithms of quantities in Fig. 3, where both sets of points follow line *A*. The other chromatogram, which had been run in air, was allowed to stand in the laboratory for 16 hours after development, then it was sprayed, the spot areas measured, and the results plotted in Fig. 3, line *B*. These graphs show that there was no advantage in developing the chromatograms in nitrogen, but that there was considerable loss of IAA on standing in light and air. These results were supported by similar experiments using the *Avena* coleoptile extension bioassay method (Table II). Chromatograms were therefore sprayed or biologically assayed immediately after drying off the developing solvent whenever possible, and, if it was necessary to store chromatograms, this was done in an atmosphere of nitrogen, in darkness at −10° C.

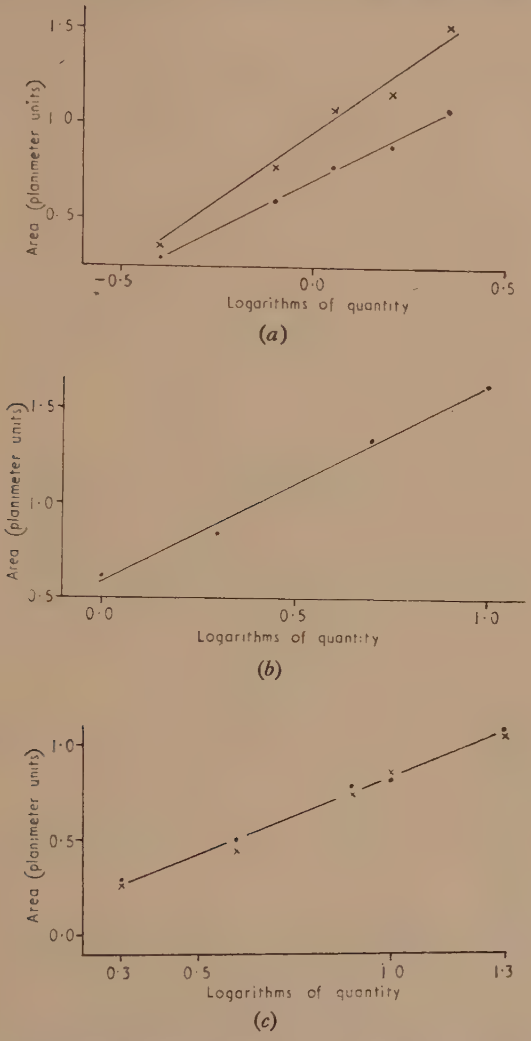

FIG. 2. The relationship between area and logarithm of quantity, for spots, of IAA originally applied with a micropipette (*a*) or a dropping pipette (*b*) and of IPA (*c*) (—×—) and IBA (*c*) (—●—), on chromatograms sprayed with ferric-perchloric reagent.

Solvents for the chromatography of indole compounds. A number of solvents have been investigated for possible use in the chromatography of IAA, IAN,

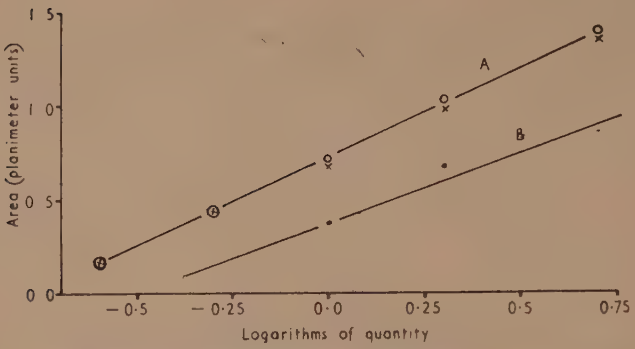

FIG. 3. The relationship between area and logarithm of quantity for spots of IAA on chromatograms developed in air (—O—), (—●—), or nitrogen (—×—) and sprayed with ferric-perchloric spray immediately (—O—), (—×—) or 16 hours (—●—) after development.

TABLE II

The elongation, expressed as the percentage extension over the original length, of Avena coleoptile sections used in the bioassay of IAA chromatograms developed in an atmosphere of air or nitrogen and bioassayed immediately or 16 hours after development. The standard elongations produced by 0·5 mg./l. IAA, added immediately before testing, are shown and the standard errors are in brackets

		Chromatograms		
		Developed in air		
Expt. No.	Standard IAA 0·5 mg./l.	Assayed 16 hrs.	Assayed immediately	Developed nitrogen, assayed immediately
7A	66 (2·5)	—	66 (1·0)	—
7B	65 (1·5)	52 (2·5)	—	—
7C	67 (1·0)	—	66 (2·5)	—
7D	—	102 (3·0)	—	120 (3·0)
7E	69 (2·0)	—	—	67 (2·5)

and gramine, which are reasonable standards for the fractions of plant extracts containing acidic, neutral, or basic substances. The results of the trials with IAN and gramine have been collected in Table III and, using the criteria of convenient R_F value and spot shape, it was decided that, of the solvents tried, 40 per cent. ethanol was most satisfactory for IAN and isopropanol: water:acetic acid::100:10:2, for gramine. The normal solvent for IAA, aqueous *iso*propanol with ammonia, gave a high R_F value for IAN and with gramine gave a tailed spot.

Alcohols other than *iso*propyl, namely ethyl, *n*-butyl, and *n*-amyl, were tried in the solvent mixture for the chromatography of IAA, IPA, and IBA. In the presence of ammonia it was found that the R_F values decrease as the carbon chain length of the alcohol increases. The reverse is the case under neutral or acid conditions.

TABLE III

The R_F and R_{IAA} values and spot shape of spots of IAN and GRM chromatographed with a number of solvents and made visible with nitrite-nitric spray

Compd.	Solvent	R_F	R_{IAA}	Spot shape
IAN	70% ethanol	0·8	—	round
	40% ethanol	0·65	—	round
	petroleum ether 60°–80° C. satd. with water	0·2	—	tailed
	ligroin satd. with water	0·55	—	tailed
	*iso*propanol : water 9 : ammonia : : 10 : 1 : 1	0·8	2·2	round
	n-butanol satd. with water and ammonia	0·75	3·2	round
GRM	*iso*propanol : water : : 10 : 1	0·55	1·6	tailed
	*iso*propanol : water : acetic acid : : 100 : 10 : 2	0·45	0·85	round

Plant materials. The plant materials examined have usually been etiolated seedlings, which were chosen for their possible use in subsequent growth studies. Other materials have been examined as the development of the investigation has demanded. They were as follows:

Broad bean (*Vicia faba* var. 'Green Leviathan') or pea (*Pisum sativum* var. 'Pilot') seeds were sterilized, soaked, and planted in moist vermiculite and were held at 22° C. in darkness. If roots were required, these were harvested after 7 days for bean and after 4 days for pea when the radicle lengths were approximately 8 cm. and 5 cm. respectively. Shoots were harvested after 12 days for bean and 10 days for pea when the etiolated shoots were approximately 15 cm. and 10 cm. high respectively. Sunflower (*Helianthus annuus* var. 'Southern Cross') seedlings were grown in moist vermiculite at 22° C. in darkness for 8 days when shoots (5 cm.) were harvested and any remaining seed-coats removed. Maize (*Zea Mays* var. 'Great White Horsetooth') roots were grown horizontally on moist filter paper at 22° C. in darkness for 3 days and were harvested when an average length of 3 cm. Maize (var. unknown) kernels at the milky stage were removed from fresh cobs harvested at the appropriate time. Potato (*Solanum tuberosum* var. 'King Edward') shoots of average height 15 cm. were harvested from tubers which had sprouted in darkness. Potato (var. 'Gladstone') tubers were taken from storage in early winter, washed, and the outer 0·5 cm. (skin) was separated from the remainder (middle). Mangold—also called field beet (*Beta vulgaris*)—root was taken from storage in early winter and diced. The cabbage and cauliflower were fresh commercial heads.

Extraction of plant material. The common method for the extraction of plant material with ether was tried in some early experiments with potato tuber, cauliflower, and etiolated pea seedlings; however, this experience made it clear that the formation of emulsions on the direct extraction of plant material with ether was too probable for the use of this method in a quantitative routine assay.

The following method of extraction, which gave a minimum of emulsion trouble, has proved satisfactory as a routine method with the particular facilities of this laboratory. The plant material was placed in a 'deep freeze' unit at —10° C. and frozen, or was boiled for 5 minutes, as soon as possible after harvesting, then was macerated in a minimum volume of absolute alcohol. After the macerated tissue had stood with alcohol at —10° C. for at least 24 hours, the alcoholic extract was removed by centrifugation and the solid residue was washed twice with fresh alcohol. The combined alcoholic extract and washings, filtered if necessary, were evaporated under reduced pressure until all of the alcohol had been removed and there remained an aqueous residue. This was acidified to pH 3 with A.R. phosphoric acid and extracted with ether for 1½ hours in a Kutscher-Steudel extractor.

Purification of extracts. The ether extract, obtained above, was shaken three times with 5 per cent. sodium bicarbonate solution and the ether layer was retained and will be referred to as the 'ether extract containing neutral substances'. To this was added the small amount of ether used to wash the aqueous alkaline extract. The latter was then acidified to pH 3 with phosphoric acid, shaken three times with ether, and the ether extract, which will be referred to as the 'ether extract containing acidic substances', was washed with a small amount of dilute phosphoric acid at pH 3. It should be emphasized that the chromatograms described in this paper are of extracts containing acidic substances and the portions of the extracts containing neutral, basic, and certain phenolic substances have not been investigated.

In some early experiments with extracts of pea shoots the ether extract containing acidic substances was further purified by evaporating to dryness, dissolving the residue in a small amount of *iso*propyl alcohol:water:acetic acid::100:30:5 and chromatographing it on a short column of Whatman's cellulose powder, with the same mixture as running solvent. Tests with synthetic IAA showed that this substance ran at the solvent front on a cellulose powder column with the above solvent. The other acidic growth substances also appeared to pass through the column readily, but some oils and waxes, which interfered with the application of extracts to chromatograms and with their development, were removed. In all but these early experiments greater extraction efficiency made this extra purification step unnecessary.

Chromatography of plant extracts. The method used for the general application of plant extracts to sheets of chromatographic paper was adapted from that of Ashby (1951) for the application of substances, in solution in ether, to agar blocks.

A sheet of chromatographic paper was stretched over a glass plate which stood on a hot plate at 35°. A small hole, 1·5 cm. diameter, in the glass plate was made to correspond with the point on the starting line where the spot of extract was desired. Directly above the hole in the plate and clamped to the surface of the paper was a short glass cylinder fitted with a side arm through which nitrogen flowed. This cylinder acted as a jacket for a dropping pipette and held it about 1 cm. from the paper surface while the extract was being applied to the paper. An extract containing the acidic substances was evaporated to dryness and the residue was dissolved in 0·5 ml. of ether (sodium dried). This solution was drawn into the dropping pipette, the outlet of which was capillary, and, with the pipette in the jacket, the extract was applied dropwise to the starting line of the chromatogram. Two further amounts of 0·5 ml. of ether were used to wash the container of the extract and the pipette and the washings were applied to the paper as before. The nitrogen hastened the evaporation of the ether and its pressure assisted in the control of the dropping rate of the extract, which was held at the rate giving the smallest starting spot area. Marker spots of IAA and, at times, IAN were run parallel with chromatograms of plant extracts.

The development and treatment after development of the chromatograms of plant extracts was the same as described earlier for synthetic compounds. From the developed and dried chromatograms the strip of paper holding the marker spots was removed and sprayed with ferric-perchloric for IAA and nitrite-nitric for IAN. The path of the extract chromatogram was marked under a weak U.V. source and a strip, 0·5 cm. wider on each side than the marked path, was cut from the paper and this was divided at 2·5-cm. intervals, parallel with the starting line. These paper rectangles were bioassayed by the coleoptile section extension method as already described.

Purification of ether. All of the ether used in these extractions was shaken with acidic ferrous sulphate solution immediately before use.

EXPERIMENTAL RESULTS

The results of the experiments, in which ether extracts containing acidic substances from plant material have been chromatographed and the chromatograms bioassayed, have been plotted as histograms of coleoptile section extension against distance from the starting line of the chromatogram. The horizontal broken lines on the figures represent the extension of controls so that coleoptile extensions greater than this represent growth promotion and those below represent inhibition. The vertical broken lines indicate the position of the solvent front, and where these are absent the solvent front was off the paper. The standard errors for the coleoptile section tests were calculated and were found to be less than 5 per cent. of the coleoptile section elongation, expressed as the percentage extension over the original length. In all cases the results shown have been confirmed with similar experiments, and for broad bean and pea, shoots and roots and potato tuber, a large number of extracts from many different samples have been chromatographed in the course of the whole investigation, always with the same results.

Young etiolated broad bean shoots were the most commonly used material in this investigation and the results of a typical experiment using 30 g. of this material are shown in Fig. 4A, where three zones of growth activity, corresponding with three areas on the chromatogram, were apparent. One zone of growth promotion of coleoptile sections corresponded with the position of the IAA marker spot and the other promoting zone had an R_F value

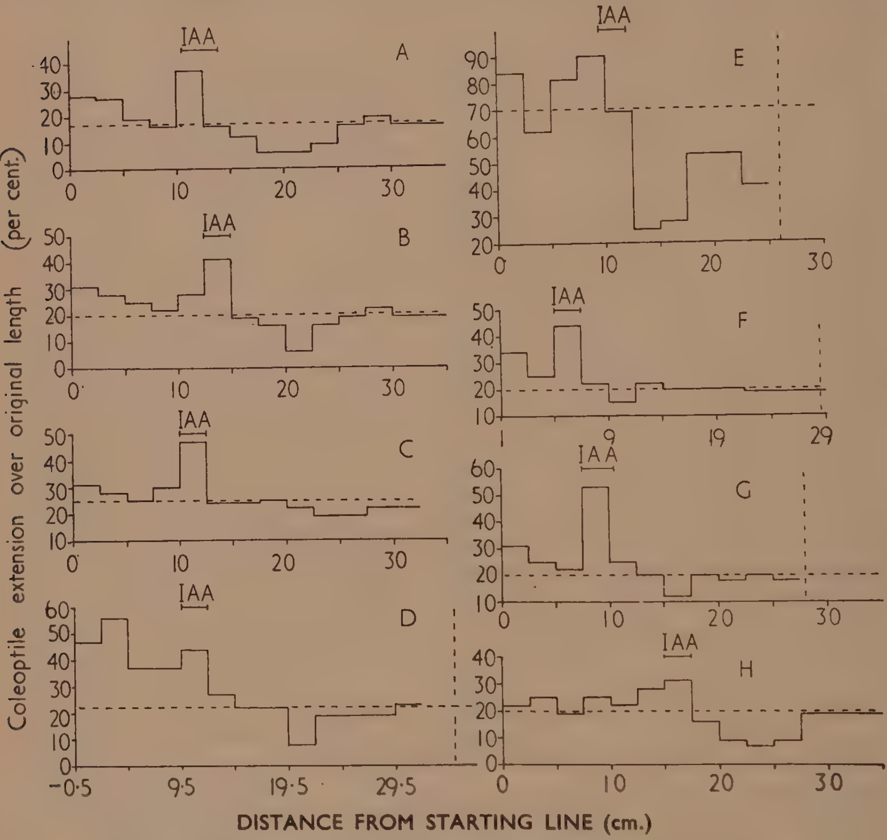

FIG. 4. Activities in coleoptile extension, shown as histograms, of squares cut from chromatograms of extracts of etiolated broad bean shoot (4A), broad bean root (4B), etiolated pea shoot (4C), pea root (4D), etiolated sunflower shoot (4E), maize root (4F), etiolated potato shoot (4G), and potato tuber 'skin' (4H). The horizontal broken lines represent the extension of controls and the vertical broken lines the solvent fronts. The IAA marker spots are shown.

less than that of IAA. A zone with R_F value greater than that of IAA showed inhibition of coleoptile section extension. This description of the results of the bioassay of the chromatogram also applied to experiments using the following broad range of plant materials: broad bean roots, 22 g. (Fig. 4B); etiolated pea shoots, 25 g. (Fig. 4C); pea roots, 35 g. (Fig. 4D); etiolated sunflower shoots, 54 g. (Fig. 4E); maize roots, 14 g. (Fig. 4F); etiolated potato shoots, 120 g. (Fig. 4G), and potato tuber skin, 50 gm. (Fig. 4H).

In Fig. 5A the results of the bioassay of an extract chromatogram of the potato tuber 'middle', 100 g., are presented. Again the three growth-active zones were apparent, but in addition to these a fourth zone, growth-promoting and lying 32·5–35 cm. from the starting line of the chromatogram, will be seen. On the chromatogram represented in Fig. 5A the growth-promoting zone of lowest R_F was not definitely present, but it was clearly apparent on a chromatogram of extract of 150 g. of potato tuber 'middle'. When a chromatogram of the ether extract containing acidic substances from 50 g. of potato skin was sprayed with nitrite-nitric spray, a spot initially dark blue and fading to light green was noted at 32·5–35 cm. from the starting line. On this chromatogram and on the one of potato 'middle' recorded in Fig. 5A the solvent had run off the paper. The results of a bioassay of a chromatogram of extract from 30 g. of maize kernels at the 'milky' stage (Fig. 5B) showed areas of growth activity similar to those found for potato tuber 'middle' extract (Fig. 5A). In the former case, however, the colour reaction on spraying of the zone 22·5–27·0 cm. from the solvent front was not found.

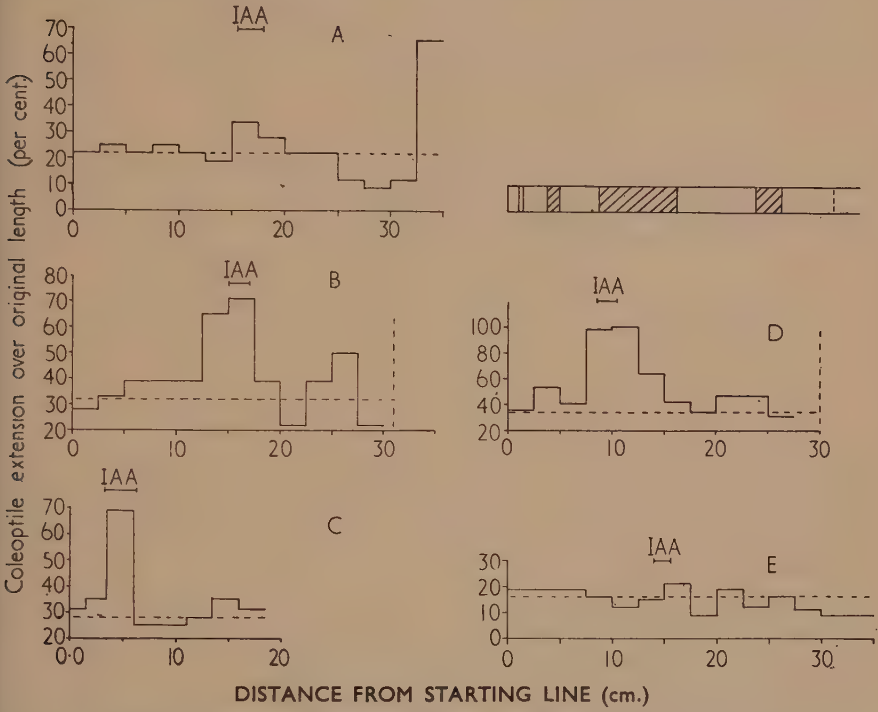

FIG. 5. Activities in coleoptile extension, shown as histograms, of squares cut from chromatograms of extracts of potato tuber 'middle' (5A), maize kernels at 'milky' stage (5B), cabbage head (5C), cauliflower head (5D), and mangold root (5E). Above histogram 5D the coloured spots on a sprayed chromatogram of cauliflower head extract are shown. Conventions as in Fig. 4.

The growth activity detected on a chromatogram from 100 g. of mangold root was weak and rather irregular, as shown in Fig. 5E.

Chromatograms of extract of cabbage have been treated in two ways. One chromatogram of extract of 3·2 g. of plant material was bioassayed together with standard amounts of IAA. The results of the bioassay, plotted in Fig. 5C, showed that the growth activity of the extract was dominated by the zone corresponding in position with IAA. Assuming that the coleoptile extension in this portion of the chromatogram (3·5–6·0 cm. from the starting line) was due to IAA, the amount of IAA on the chromatogram and hence its concentration in cabbage was determined using the linear relationship between coleoptile extension and IAA concentration (Table IV, Chromatogram 1). Three other chromatograms of cabbage extract were run, together with standard amounts of IAA, and the whole of the sheets were sprayed with ferric-perchloric spray. The spots of IAA standards, and the spots on the extract chromatograms corresponding in colour and position with IAA, were marked and their areas measured. On chromatograms of cabbage extract developed in an alkaline atmosphere, two spots with the same colour, on spraying, as IAA were detected, one with R_F value the same as that of the IAA marker spot and the other approximately 0·1 R_F units below it. The R_F of IAA suggested that the developing conditions were very weakly alkaline and that as a result of this the IAA may be running partly as an ion and partly as an undissociated molecule. This theory received support when only one coloured spot was observed on a chromatogram run under acidic conditions, when all of the IAA would run in the undissociated form. Chromatograms 3 and 4 in Table IV were cases of IAA running as two spots and the areas of both spots were measured and summed. Chromatogram 2 was developed in acidic conditions and one coloured spot, corresponding in position with an IAA marker, was observed and its area was measured. From these area measurements and those of IAA standard spots, the concentrations of IAA in the cabbage were found, and in Table IV these are compared with the concentration determined by bioassay and a reasonable agreement will be noted. Chromatograms of an extract of cauliflower head, 100 g., were bioassayed or sprayed with ferric-perchloric and the results of both tests are given in Fig. 5D. In the growth tests three zones of growth promotion, corresponding with three zones which gave a similar crimson colour on a chromatogram sprayed with ferric-perchloric, were detected.

TABLE IV

Concentrations of IAA, in extracts of cabbage, calculated from the growth activity or the area of spots, on extract chromatograms, compared with growth activities and areas obtained with known amounts of IAA

	Method of assay	Concentration IAA [mg./Kg.]
Chromatogram 1	Biological	0·054
,, 2	Area	0·063
,, 3	,,	0·051
,, 4	,,	0·059

In addition to extracts of whole broad bean shoots, in some experiments the acidic growth substances occurring in extracts of the various parts of the shoot were investigated. In one experiment a large number of etiolated broad bean seedlings, 15 cm. high, were divided into four parts and yielded: apical bud, 30 g.; stem between the apical bud and the first lateral bud, 106 g.; lateral bud including about 0·5 cm. of stem, 38 g., and stem below the first lateral bud, 138 g. An extract of each part was prepared and portions of these extracts corresponding with 10 g. of apical bud, 30 g. of upper stem, 12 g. of lateral bud, and 40 g. of lower stem were chromatographed, and the results of the bioassays are shown in Fig. 6, where the portions of the seedlings treated are also indicated. The three growth-active zones noted in extracts of whole seedlings were present in all extracts, but the relative importance of the zones in each extract were of interest.

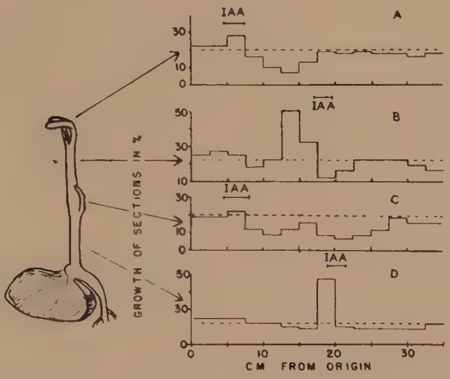

FIG. 6. Activities in coleoptile extension, shown as histograms, of squares cut from chromatograms of extracts of etiolated broad bean shoots divided as shown into apical bud (A), stem between apical bud and first lateral bud (B), first lateral bud (C), and stem below first lateral bud (D). Conventions as in Fig. 4.

The broad bean root has been studied in two sections, 0–2 cm. and 2–4 cm. from the tip, and the roots that were divided yielded 60 g. of the 0–2-cm. section and 110 g. of the 2–4-cm. section. One-quarter of the extract from each section was chromatographed and the results of the bioassay, given in Figs. 7A and 7B, show that there was no significant qualitative or quantitative difference in the growth substances contained in them, nor were they different from the growth substances in the whole root.

Two methods of pretreatment of plant materials before extraction were compared when two identical samples of etiolated broad bean shoots, 30 g., were respectively (*a*) boiled with water for 5 minutes and (*b*) frozen before maceration with alcohol. The mixtures were held at −10° C. for 7 days, after which the standard procedure of extraction, chromatography, and bioassay was followed for both examples. The results of the bioassay, as shown in

Figs. 7C and 7D, were identical and indicate that, at least in this material, both methods of enzyme activation can be regarded as relatively unobjectionable.

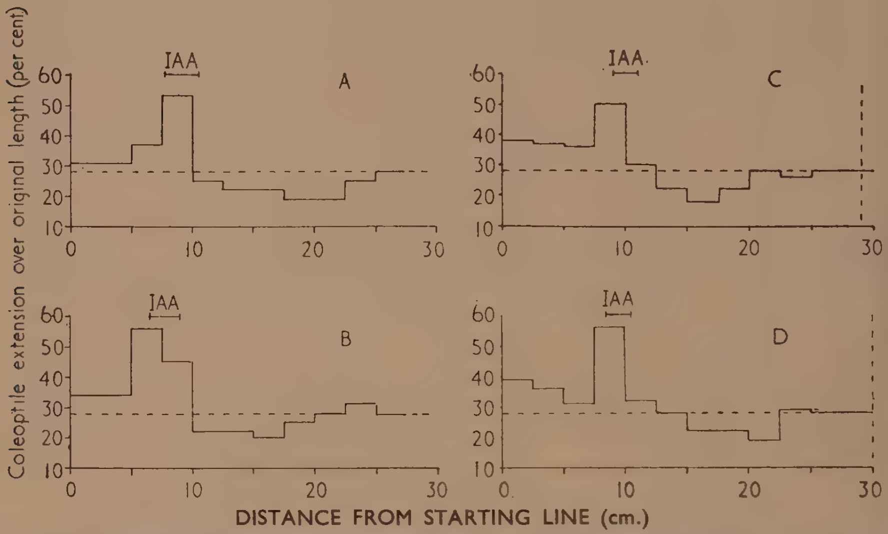

FIG. 7. As in Fig. 4, for extracts of broad bean roots divided into two sections, 0·0–2·0 cm. (7A) and 2·0–4·0 cm. (7B) from the tip; and for extracts of etiolated broad bean shoots, frozen (7C) or boiled (7D) before extraction.

DISCUSSION

With rare exceptions, three zones, active in coleoptile section growth, were detected on chromatograms of all plant extracts studied. One zone corresponded with the position of the IAA marker spot and in all cases promoted the growth of the coleoptile sections. With a plant material showing high activity in this zone, namely cabbage, a coloured spot was obtained on a sprayed extract chromatogram, which corresponded in colour and position, in two solvent mixtures, with IAA. In addition, quantitative determinations of IAA, based on the areas of these spots and on the activity in coleoptile extension of the zone, were in reasonable agreement. Thus it is almost certain that the growth activity of the zone corresponding in position with the IAA marker spot is due to IAA and to IAA alone. Further evidence from growth *v.* concentration curves will be presented in Part II of this series. The substances responsible for the growth activity of the other two zones are not known, and assuming that the zone with R_F value less than that of IAA, corresponds with one substance, the term accelerator α has been used for it and on the same basis the term inhibitor β is suggested for the component with R_F slightly greater than IAA.

IAA, accelerator α (α), and inhibitor β (β) have been shown to occur in the following wide variety of plant materials: broad bean shoots and roots, pea shoots and roots, sunflower shoots, maize roots and immature kernels, and potato shoots and tubers. To the results presented here may be added those of Luckwill (1952), IAA and inhibitor β in broccoli leaves and asparagus fruits and β in pear fruit buds, grape seeds, tomato stamens, and tomato stem tips; Hancock and Barlow (1953), IAA, α, and β in apple leaves; Lexander (1953), IAA, α, and β in wheat roots; Thimann and Stowe (1953), IAA and possibly α in sweet corn seed; Terpstra (1953), IAA and possibly α in *Avena* coleoptile tips; and Kefford (1954, Part III), IAA, α, and β in the rhizomes of *Aegopodium podagraria*. All of these workers obtained their results by the bioassay of chromatograms.

A striking feature of the results of the assay of root and shoot extracts is the great similarity between the growth-substance contents of seedling roots and etiolated shoots. This point is seen very well in Figs. 4A and 4B, where the results of bioassays of chromatograms of broad bean shoot, 30 g., and broad bean root, 22 g., have been placed together. Since in many growth reactions, which are considered to be hormone-controlled, shoots and roots are opposites, this similarity in growth-substance pattern is unexpected unless the physiological reactions of shoots and roots with the growth substances are opposites. If the latter is the case one would have to postulate that the IAA detected in the root is acting as a brake on cell elongation. This has been put forward before as a means of shoot-root interaction.

On chromatograms of extracts of potato tuber 'middle' (Fig. 5A) a fourth growth-active zone, close to the solvent front, was observed as well as the zones due to α, IAA, and β. This fourth zone corresponded in position with a spot, on a chromatogram of potato tuber skin extract, which, when sprayed with nitrite-nitric spray, gave the characteristic colour reaction of IAN. A spot of IAN, when sprayed with nitrite-nitric, initially goes dark blue then fades to light green. The activity of this fourth zone is probably due to IAN, and its identity and presence in an extract fraction, considered to contain only acidic substances, will be discussed further in connexion with extracts of *Aegopodium* rhizomes, in which IAN was first detected in this laboratory. The absence of an IAN zone on the chromatogram of potato tuber skin extract (Fig. 4H) was probably due to this zone moving off the paper, since the solvent front was off the paper. The IAN, which apparently occurs in relatively large quantities in the above tissues and in members of Cruciferae (Jones *et al.*, 1952; Bentley and Bickle, 1953) and which may be readily converted to IAA under special circumstances (Thimann, 1953), has been suggested by Thimann (1954) as providing bacteria with a possible source of large local concentrations of IAA that could produce deformities in an infected plant.

On the chromatogram of extract from 'milky' maize kernels (Fig. 5B), in addition to α, IAA, and β a zone of growth promotion was found at 22·5–27 cm. from the starting line, where IAN could be expected to run; but, unfortunately, the colour given by this zone on spraying was not known. Redemann *et al.* (1951) obtained a high yield of ethyl indole-3-acetate (EIA) in an ethanolic extraction of immature maize kernels at room temperature, but, as Henbest *et al.* (1953) have pointed out, the EIA could be an artifact, enzymatically produced during the extraction with ethanol. Good evidence has been presented (Teubner, 1953) for the occurrence of EIA in apple endosperm and in this case the extracting solvent was ether. With the chromatographic solvent used in the present investigation EIA has an R_F value just greater than that of IAN; in fact Stowe and Thimann (1954) have shown that in this solvent most neutral indole compounds congregate near the solvent front. Hence, although the presence of IAN in potato tuber was established by the characteristic blue to green colour reaction with nitrite-nitric spray (EIA gives a yellow colour with this reagent), the possibility of the zone of growth promotion near the solvent front on the maize kernel chromatogram being due to EIA must be considered. For this to be the case EIA would have to leak through the purification process that gave the ether extract containing acidic substances, which was chromatographed. Although this leakage occurred with IAN (Bennet-Clark and Kefford, 1953) it does not seem likely that the large amounts of EIA, necessary to give the high growth promotions that have been found in the right position on maize kernel chromatograms, would pass through.

The major acidic growth substance in cauliflower head was found to be IAA (Fig. 5 D), but two minor growth promoters which also gave crimson-coloured spots on spraying were detected. The spot with R_F value less than that of IAA could be IPyA, for Stowe and Thimann (1953) found that, in this solvent, IPyA had an R_F about half that of IAA and the amount, necessary to give a good colour as a spot on a chromatogram sprayed with ferric-perchloric spray, produced growth promotion in coleoptile sections under the present conditions. Weller *et al.* (1954) have made a chromatographic study of the indole compounds in cauliflower head.

The experiment in which the apical bud, the lateral bud, and the stem of broad bean seedlings were studied (Fig. 6) showed that although α, IAA, and β were probably present in all the shoot parts, in the extracts of stem and lateral bud a predominance of one substance was apparent. Fig. 6 A shows that the apical bud contained α, IAA, and β in similar proportions to those found in the whole shoot, Fig. 4 A. The lateral bud extract, Fig. 6 C, however, showed a predominance of β and the stem sections, Figs. 6 B and 6 D, a predominance of IAA. These results would suggest that in the stem of the seedling, which is the part that is elongating rapidly, IAA may be the controlling growth substance and in the lateral bud where elongation growth is inhibited, β controls the growth. It could be that IAA, which is produced in the apex and travels down the stem, is converted into or causes the production of inhibitor β which accumulates in and inhibits the growth of the lateral bud and is thus responsible for the phenomenon of apical dominance. The experiments of Skoog (1939) showing that growth of isolated lateral buds was inhibited by IAA would suggest that the conversion of IAA to β would have to occur within the buds. Some evidence for bud inhibition being due to an inhibitor produced from auxin was given by Snow (1940), and Thimann (1952) supports this type of explanation for apical dominance.

Potato tuber and mangold were investigated as possible large-scale sources of α and β. The skin of potato proved to be a good source; thus mangold, the chromatogram of which gave weak and irregular growth activity, Fig. 4 H, was not investigated further. The potato tuber and in particular the tuber 'skin' was chosen as a large-scale source of inhibitor β because this substance occurred in large amounts in the tissue and appeared to be the predominating growth substance. This finding supported Hemberg's (1947, 1949) theory that potato tubers were held in dormancy by the action of an inhibitory substance.

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The Paper-Chromatography of the Pyridine- and Piperidine-Carboxylic Acids

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SUMMARY

The R_F values in three conditions, and measured under carefully controlled conditions, are given in these solvent systems for pyridine 2-, 3-, and 4-carboxylic acids and for piperidine 2-, 3-, and 4-carboxylic acids.

Certain anomalies in these R_F values are discussed.

In work leading to the identification of the amino-acids responsible for two ninhydrin-reacting spots on two-dimensional paper chromatograms of apple-fruit extracts (Hulme and Arthington, 1952) a study was made of the behaviour of the cyclic pyridine- and piperidine-carboxylic acids. The results obtained are described below, since it appears possible that all these acids may be present in biological material.

EXPERIMENTAL

Chromatographic technique. Chromatograms were run by downward flow in glass tanks in a constant-temperature room at 20° C. All solvents were prepared at least 48 hours before use and the papers were equilibrated in the tanks for 24 hours before flow of solvent was commenced. The solvents used were *A*, *n*-butanol-acetic acid-water (4:1:5 v/v); *B*, propanol-ammonia (sp. gr. 0·880)-water (60:30:10 v/v); *C*, *n*-butanol-pyridine-water (equal proportions v/v). Whatman No. 1 'chromatographic' paper was used throughout (17·5 cm. × 58 cm.). The spots were applied 2·5 cm. apart to the starting line (8 cm. from the end of the paper) by means of a micropipette, 5 μl. of a solution containing 3 mg/ml. being taken in each case. After the chromatograms had been run they were dried in a special oven with rapid air circulation at 100° C. The sprays used for revealing the position of the acids were (1) ninhydrin—0·1 per cent. in a mixture of equal parts of ethanol and butanol containing 0·02 per cent. stannous chloride; (2) isatin—0·2 per cent. in butanol containing 4 per cent. acetic acid; (3) bromo-phenol blue—0·5 per cent. in 50 per cent. ethanol, made faintly alkaline with 0·1 N. NaOH. This indicator spray was used for revealing the pyridine-carboxylic acids, as acids. After spraying with sprays (1) or (2) the papers were heated to 110° C. for 5 minutes.

Source of acids. Commercial preparations of the three pyridine-carboxylic acids (picolinic, nicotinic, and *iso*nicotinic) were used. Samples of the three piperidine-carboxylic acids (pipecolic, nipecotic, and *iso*nipecotic acids) were synthesized, as the hydrochlorides, from the corresponding pyridine-carboxylic acids by hydrogenation at 2 atmospheres pressure in the presence of

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Adams catalyst. This is essentially the method described by Stevens and Ellman (1950) for the preparation of piperidine-2-carboxylic acid. The hydrochlorides of the three acids (2-, 3-, and 4-COOH) had the following m.p.s. (corrected) 256–260° C., 239–242° C., 293–294° C.

RESULTS

Although no great reliance is placed on the absolute R_F values of amino-acids (the elaborate precautions necessary to obtain exactly reproducible values are not suitable for routine work), relative values obtained with acids run side by side on the same chromatogram yield reproducible ΔR_M values (Bate-Smith and Westall, 1950) for a series of acids. The R_F and R_M values obtained for the pyridine- and piperidine-carboxylic acids in three solvents are shown in Table I.

TABLE I

R_F and R_M values of pyridine- and piperidine-carboxylic acids in three solvent systems, A, B, and C given in text

	A				B			C		
	R_F	R_M	ΔR_M		R_F	R_M	ΔR_M	R_F	R_M	ΔR_M
Pyridine-2-COOH	0·42	+0·14			0·60	−0·18		0·48	+0·03	
			0·47				0·13			0·13
Pyridine-3-COOH	0·68	−0·33		0·19	0·67	−0·31		0·56	−0·10	
			0·28				0·06			0·06
Pyridine-4-COOH	0·53	−0·05			0·70	−0·37		0·59	−0·16	
Piperidine-2-COOH	0·31	+0·35			0·63	−0·23		0·45	+0·08	
			0·04				0·17			0·21
Piperidine-3-COOH	0·29	+0·39			0·535	−0·06		0·34	+0·29	
			0·04				0·08			0·10
Piperidine-4-COOH	0·27	+0·43			0·49	+0·02		0·29	+0·39	

A striking characteristic of the piperidine-carboxylic acids is the bright red fluorescence which their ninhydrin compounds give in ultra-violet light. The colours of the three acids after treatment with ninhydrin in visible light are shown in Table II. The bright blue colour of piperidine-4-carboxylic acid while the paper was still hot was very striking. All three acids give a bright bluish-green (as distinct from the true blue of the pyrrolidine-carboxylic acids) with isatin, the colour appearing within a very short time of heating. Both series of acids tended to streak in acid solvents but gave excellent round spots in the basic solvent systems, the colours with ninhydrin (piperidine series only) being particularly intense after travelling in solvent system *C*.

TABLE II

Colours of the ninhydrin compounds of the three piperidine-carboxylic acids

	Colour in daylight	
Acid	While paper still hot	When paper has cooled
Piperidine-2-COOH	Blue-purple	Rose-purple
Piperidine-3-COOH	Purple Blue-grey	Blue-purple with tinge of purple
Piperidine-4-COOH	Blue	Blue-grey

The piperidine ring, unlike the 'aromatic' pyridine ring, is fully saturated and possesses no double bonds to bring into play mesomeric effects. Piperidine in fact behaves like a typical aliphatic amine and the piperidine-monocarboxylic acids should be expected to behave like aliphatic imino-acids. It will be seen from Table I that the R_F values in acid and basic solvent systems of these acids decrease progressively as the carboxyl group moves round the piperidine ring from the 2- to the 4- position. In acid solvents ΔR_M for the shift from position 2 to 3 and from 3 to 4 is identical, whereas in both basic solvents ΔR_M for the first shift is twice that for the second shift. This difference may be due to the fact that in the presence of acetic acid (acid solvent systems) the ionization of the carboxyl group will be completely suppressed whereas suppression of the ionization of the strongly basic piperidine group will not be fully achieved in the presence of the weaker bases ammonia and pyridine (basic solvents).

The R_F values for piperidine-2- and piperidine-3-carboxylic acids are of the same order in the respective solvent systems as those found by Morrison (1953).

With the pyridine-carboxylic acids the situation is more complicated owing to the unsaturated nature of the pyridine ring. In acid solvent systems, although ionization of the carboxyl group is suppressed, conditions are favourable for salt formation. This would not, however, be expected to influence R_F values. It seems more likely that the anomalous behaviour of the three pyridine-monocarboxylic acids in acid (COOH group un-ionized) solvents (Table I) is due to the same cause (electronic disturbances) as the similar general reactive differences between 2- and 4- on the one hand and 3- substituted derivatives of pyridine (cf. *o*-, *m*-, and *p*- substituted derivatives of benzene). Electron shifts (and resonance 'stabilization') will be different when the carboxyl group is un-ionized (ionogenic structure) than when ionized (ionic structure). It is interesting that the mono-nitrobenzoic acids behave similarly to the pyridine acids as regards R_F values in acidic and basic solvents; similar electronic disturbances are possible in both the pyridine and nitrobenzene 'nuclei' of these two series of acids.

With the pyridine acids, ΔR_M is the same between the 2- and 4- positions in *both* acidic and basic solvents; pyridine is a much weaker base than either piperidine or ammonia.

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Interrelationships between Light Intensity, Temperature, and the Physiological Effects of 2:4-Dichlorophenoxyacetic Acid on the Growth of *Lemna minor*

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SUMMARY

The effects of light intensity and temperature on the changes in growth and development induced by sodium 2:4-dichlorophenoxyacetate have been studied in multifactorial experiments. Random samples from a clonal population of *Lemna minor* were subjected to three intensities (700, 275, and 180 foot-candles) and three temperatures (30°, 25°, and 20° C.), while varying concentrations of the growth regulator were added to a phosphate-buffered culture solution (pH 5·1). There were up to five sampling occasions when the dry weights and, for some purposes, the total frond areas were determined. The replication was at least twofold, and the largest experiment involved 720 dry-weight determinations.

At each combination of light and temperature, sublethal concentrations of the growth regulator induced cumulative reductions in the relative growth rate and the order of the depression increased progressively with concentration—3, 9, 27, and 45 p.p.m. Because of the cumulative effects and the great differences in the growth rates of the controls under the nine combinations of light and temperature, the statistical analysis of the data was involved. Employing two different approaches it was concluded that the relative depression in the growth rate increased with temperature while the growth of plants receiving the highest intensity was slightly more inhibited.

At a concentration (27 p.p.m.) which reduced both the relative growth and frond area, over all combinations of light intensity and temperature the net assimilation rate during the periods 0–2 and 2–4 days was depressed by 2 and 12 per cent. respectively. There was some indication that the depression was larger at the highest temperature in the second period.

At greatly increased concentrations the fronds exhibited chlorotic symptoms, and the concentrations required to produce a standard degree of chlorosis at the end of 3 days were determined under twelve combinations of light intensity and temperature. It was found that varying the intensity during the time of treatment was without effect, but that there was a highly significant influence of the light level prior to treatment: the lower the intensity the smaller was the concentration subsequently required to induce chlorosis. Likewise, with a rise in temperature the magnitude of the equi-effective concentration was reduced.

These interactions are discussed in relation to changes in the ratio of frond area to frond weight, the mechanism of uptake, and possible physiological effects at cell level.

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INTRODUCTION

THE initial field investigations relating to the development of 2:4-dichlorophenoxyacetic acid and allied compounds as selective herbicides soon established that the physiological effects were linked with environmental conditions, but when the present research was started in 1950 the precise roles of such factors as temperature and light intensity had not been elucidated. Marth and Davis (1945) had established in greenhouse experiments that there was a positive relationship between temperature and toxicity, while Aslander (1950) had reached the same conclusion from observations in the field. However, in these investigations the changes in temperature were not independent of changes in other factors, but Kelly (1949) in more critical laboratory experiments with 2:4-dichlorophenoxyacetic acid confirmed that the level of temperature both before and after application of the growth regulator influenced the activity, where this was estimated in terms of death or visual symptoms.

Other laboratory experiments (Weintraub and Brown, 1950*b*) indicated that on an area basis the inhibition of the growth of the terminal leaves of bean plants was independent of temperature when the compound was placed on the bud. In contrast, if the primary leaves were treated the inhibition of the growth of the bud was proportional to temperature, and on the basis of these results the authors concluded that temperature operated through an effect on translocation.

In the field it had been reported by Thimann (1947) and Egler (1950) that plants receiving full daylight were slightly more susceptible than shaded plants to the action of 2:4-dichlorophenoxyacetic acid, but others, such as Hitchcock and Zimmerman (1948), claimed that a greater mortality occurred under shade conditions.

When the growth regulator was applied to the leaves of snap-beans, curvature of the stem only resulted if the plants were exposed to a combination of an adequate supply of carbon dioxide and a high light intensity (Mitchell and Brown, 1946). These investigators, together with Weaver and de Rose (1946), demonstrated that the material moving out of the leaf which induced stem curvature could be transported upwards through non-living xylem but not downwards through steamed petioles. For *Eichornia crassipes* and the kidney bean Penfound and Minyard (1947) claimed that applications of the butyl ester in kerosene to the leaves induced a greater general toxicity if the plants were shaded but that the development of epinastic symptoms was not influenced by light intensity. Such oil solutions will spread over the leaf surface and probably down the petiole, and Linder, Brown, and Mitchell (1949) found that when precautions were taken to prevent this spread by placing the solutions within a gelatine ring on the leaf no epinasty developed in the stems of bean plants grown in total darkness. This lack of response could not be related to a failure of the phenoxyacetic acid to penetrate leaves, for Rice (1948) had established that the rate of entry was slightly greater in darkness than at intensities of 100–900 foot candles.

Rohrbaugh and Rice (1949) and Weintraub and Brown (1950*a*) were the first to establish that the transport out of leaves was associated with the translocation of carbohydrates. Thus, when plants were kept in the dark, physiological effects in the stem could only be brought about if the leaves treated with the growth regulator were immersed in sugar solutions. Linder, Mitchell, and Wood (1950) concluded that for both 2:4-dichloro- and 2:4-dichloro-5-iodo-phenoxyacetic acids formative effects in the stem occurred only when the light intensity was favourable for photosynthesis and the transport of metabolites out of the treated leaf.

Thus, on the basis of these published results it seemed that though the role of light in the translocation of 2:4-dichlorophenoxyacetic acid had been clearly established, little information was available as to the influence of temperature on the induced changes in growth, while the data for the over-all effects of varying light intensity were imprecise and the findings contradictory. This paper gives some account of the interrelationship between temperature, light intensity, and varying concentrations of 2:4-dichlorophenoxyacetic acid in the nutrient solution on the growth and development of the aquatic higher plant, *Lemna minor*. A second paper will describe a complementary study of the influence of shading on the changes in growth of *Helianthus annuus* following on the application of the same compound to the whole shoot or to individual leaves.

EXPERIMENTAL METHODS

The experimental technique has been described in some detail in a previous paper (Blackman and Robertson-Cuninghame, 1954) and, in consequence, only a brief description is demanded. The source of the *L. minor* was a clonal population which had been made from a single frond selection in 1949. The culture solution, containing trace elements, was a modified form of one of the solutions found satisfactory by Steinberg (1941) while the pH was adjusted to the optimal value of 5·1. The plants were grown in 250-ml. 'Hysil' beakers which were placed in waterbaths (4 × 1 ft.) of which the temperature was controlled thermostatically to ±0·5° C., while to maintain maximum uniformity between baths at the same temperature the water was circulated by a pump. The sources of light were banks of 'Osram' daylight fluorescent tubes fitted with reflectors, and the intensity was varied either by increasing the number of tubes or by altering the height above the bath. The light intensity at the surface of the *Lemna* fronds was estimated and checked weekly by means of a selenium photo-electric cell which in turn was calibrated and checked from time to time against a 1,000-watt A1 Osram projector lamp calibrated to within 2 per cent. of the standards of the National Physical Laboratory. It is realized that the two light sources differed in the spectral distribution of the light energy, but nevertheless the readings give an accurate measure of the relative differences in light intensity.

In order to maintain the pH relatively constant (±0·2 units) and to keep down algal infection the fronds after washing were transferred to fresh culture solution every 2 days. At the beginning of an experiment 10–25 fronds were placed in each beaker, and since the distribution and mean number of fronds per plant was correlated with light intensity, within each light treatment it was arranged that the proportions of plants with different frond numbers was the same in each beaker as in the population from which the samples were drawn.

Under conditions where the growth rate was high or the experiments of long duration, to prevent overcrowding the population was divided between two or more beakers. Alternatively, when this procedure was impracticable because of the number of beakers involved, at regular intervals the frond number was reduced by an appropriate fraction and allowances made for these reductions on the basis of the number and weight of the final sample.

The samples for weight determinations after rinsing in distilled water were placed in weighing-bottles and dried over silica gel for 90 minutes at 50° C. and a pressure of 1 cm. of mercury. The area of the fronds was derived by illuminating the beaker from below and photographing both the fronds and a scale of known area from above. The developed negatives were then projected through an enlarger on to a sheet of paper, the outline of the fronds and scale traced, and the areas subsequently determined with a planimeter. The accuracy of the method was high, the coefficient of variation being 2·6 per cent.

In the investigations relating to the effects on growth and development the range of concentrations was restricted to levels which retarded growth but did not during the course of the experiments produce symptoms of severe injury. In other complementary experiments a study was made of concentrations which were great enough to cause marked physiological disturbances culminating in death. As a measure of these toxic effects the degree of chlorosis developed after relatively short exposures has several advantages (Blackman, 1952) and the procedure adopted was as follows. A standard number of fronds was placed in a series of beakers containing varying concentrations of the growth regulator (buffered to pH 5·1) and the level of chlorosis assessed at the end of 3 days. As the relationship between this phytotoxic effect and the concentration is sigmoid, the criteria of measurement selected should be such that the data are quantal, since this allows of the most critical appraisement by the methods of probit analysis (Sampford, 1952). Thus the criterion adopted was to estimate the percentage of chlorotic fronds, and for this purpose all those fronds which were 'bleached' from the distal end to the regenerating portion were counted as chlorotic.

EXPERIMENTAL RESULTS

The effects of light intensity and temperature on growth and development

Relative growth rate. After preliminary experiments had been carried out to ascertain the general nature of the response of *L. minor* to a range of concentrations of sodium 2:4-dichlorophenoxyacetate in the culture solution, a comprehensive multifactorial experiment was undertaken. The design involved three light intensities (700, 275, and 180 foot-candles), three temperatures (20°, 25°, 30° C.), five concentrations (0, 3, 9, 27, and 45 p.p.m.) and, apart from the initial sampling, four further sampling occasions. Because of the size of the experiment, i.e. 720 individual beakers, it had to be divided into three parts, each consisting of one temperature but all the other variables.

In the first part of the experiment, that is at 30° C., there were three subdivisions separated by time: in each of the first two of these subdivisions there were two treatments (control and one concentration), while the third consisted of three treatments (control and the two remaining concentrations). Four replicates were harvested at the commencement and four replicates of each treatment were harvested at intervals of 5, 7, 9, and 11 days.

When the concentration was 45 p.p.m. and the light intensity was low, several fronds died during the first 48 hours. Since these plants had not produced daughter fronds before they died, their remains were removed and the number recorded. The weight that such a culture would have attained had all the fronds grown at the same rate as those that survived was calculated on a *pro rata* basis. It is possible that calculating the weight in this way is not entirely justified, so caution must be used in the interpretation of results obtained with this particular concentration.

The design of the experiment at this temperature was not entirely satisfactory, since before the results of the three subdivisions could be combined it was necessary to demonstrate that there was no significant difference between the subdivisions. This statistical analysis showed that for the controls at any one intensity there was no significant difference between the regression coefficients of growth on time for the three sections. The design of the experiment was improved for the parts carried out at 20° C. and at 25° C. At each temperature one half of the replicates of all the treatments were arranged in each of two blocks which were separated in time. For a more precise estimate of the initial weight, eight beakers were harvested in each block at each light intensity. Subsequent harvests of two replicates per treatment were made after 7, 9, 11, and 13 days. Statistical analysis revealed that for the controls at each light intensity there was no significant deviation from a linear relationship between time and the logarithm of the weights: nor was there any difference between blocks.

The mean weight of cultures treated with the phenoxyacetic acid was less than that of the corresponding controls under any combination of light intensity and temperature. The actual reductions of the logarithms of weight were greater at high light intensities, that is, under conditions when the growth rate of control plants was high. This correlation between the rate of growth and the magnitude of the depression raised a problem of interpretation. It was first necessary to determine whether there was a direct effect of light intensity on response or whether the actual difference in depression was due entirely to differences in the rate of growth. Since the effect of the growth regulator, in terms of weight, increased progressively with time, it was to be expected that if the growth rate was increased then the order of depression would also be increased.

From Table I it is apparent that the rate of growth differed considerably between light intensities and, to a smaller degree, between temperatures. Therefore, in order to compare the relative effectiveness of the growth regulator under different environmental conditions it was necessary to relate the changes in weight of the treated cultures to those of the corresponding controls. In order to do this a conversion procedure was adopted so that the adjusted logarithm of the weight of the controls for all environments could be made equal at any one time. Thus, the logarithm of the initial mean weight for an individual treatment was subtracted from the logarithm of each observed weight at a later time (time t) to give $\log W_t - \log W_0$. This difference in the logarithms of weight was divided by the regression coefficient of the appropriate control to give the expression $(1/b)(\log W_t - \log W_0)$. This final expression is referred to as the adjusted logarithmic growth. It follows, from

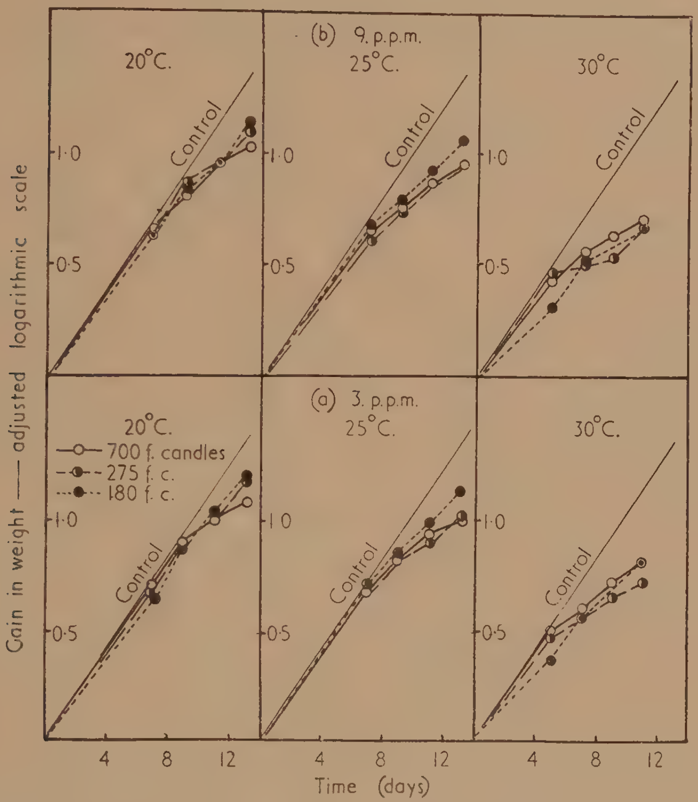

FIG. 1. The effects of light and temperature on the magnitude of the depression in the growth rate induced by sodium 2:4-dichlorophenoxyacetate at concentrations of 3 and 9 p.p.m. The changes in weight with time have been adjusted to allow for the varying growth rates of the controls under the different experimental conditions. For details see text.

the equation for the regression of the logarithm of weight on time

$$(\log W_t = \log W_0 + bt),$$

that the value of the adjusted logarithmic growth of a control culture at any time was independent of the environmental conditions.

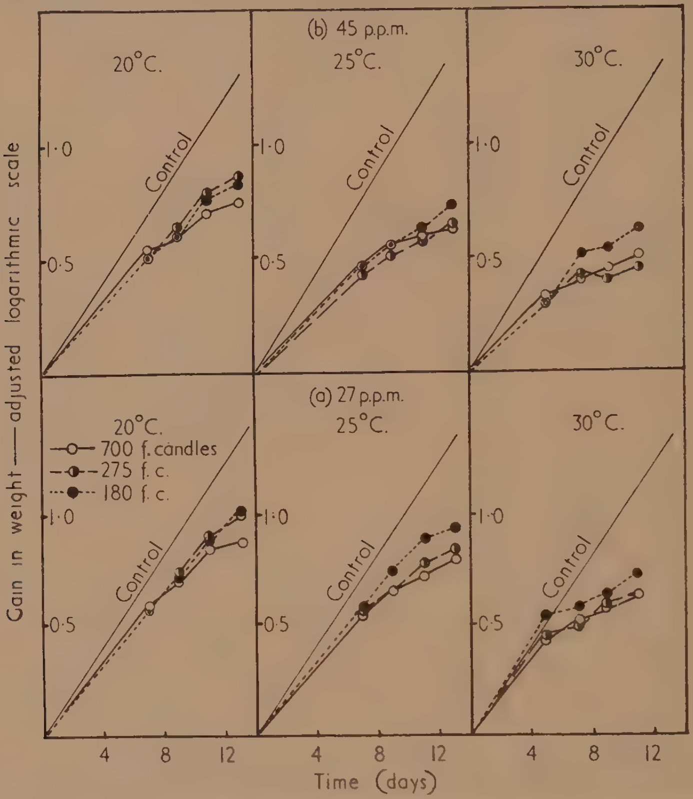

FIG. 2. The effects of light and temperature on the magnitude of the depression in the growth rate induced by sodium 2:4-dichlorophenoxyacetate at concentrations of 27 and 45 p.p.m. The changes in weight with time have been adjusted to allow for the varying growth rates of the controls under the different experimental conditions. For details see text.

An estimate of the effectiveness of each concentration of the growth regulator at any one temperature and light intensity was made by determining the

TABLE I

The effects of varying light intensity and temperature on the coefficient of the regression of log. weight on time for control cultures of L. minor

Light intensity (foot-candles)	Regression coefficient		
	Temperature		
	30° C.	25° C.	20° C.
700	0·1495±0·0018	0·1347±0·0017	0·1087±0·0014
275	0·0884±0·0017	0·1033±0·0011	0·0727±0·0017
180	0·0617±0·0019	0·0727±0·0015	0·0584±0·0025

actual amounts by which the values of adjusted logarithmic growth were depressed below that of the corresponding control. The results at each of the three light intensities have been plotted against time for each of the three temperatures in Figs. 1 and 2.

Considering all the figures, the over-all effect of temperature is clearly evident. At any one light intensity and for each concentration of the growth regulator the deviation, below the regression line of the control, increases as the temperature is raised from 20° C. to 30° C. It should, however, be noted, when making such comparisons, that the duration of the experimental period at 30° C. is 2 days less. One slight departure from the general effect of temperature occurs at the highest concentration (45 p.p.m.): here, at the lowest light intensity, the magnitude of the depression at 30° C. is not seemingly greater than that at 20° C. (Fig. 2). However, it has already been pointed out that, since adjustments had been made to compensate for the number of fronds that died at the highest concentration at 30° C., such exceptions to the over-all effect are to be expected.

The effect of light intensity on the retardation in growth caused by the phenoxyacetic acid is less clearly defined: at both 25° C. and 30° C. there is an over-all tendency for the depression to be greater at the two higher light intensities. At 20° C. the depressions at the two lower intensities are less than that at the highest intensity. There are certain minor exceptions to the general trends at 30° C., the two lowest concentrations inducing approximately equal effects at all levels of light intensity.

In the further analysis of the interrelationships between light intensity, temperature, and concentrations another approach was to fit a generalized growth curve for any one combination of light intensity, temperature, and concentration. The growth curve was based on the equation

$$\log_e \text{weight} = K - \log_e(Z + e^{-Bt}),$$

in which the parameters may be interpreted as follows. K represents the general weight so that when B and Z are fixed K depends only on the original weight. B is the generalized growth rate and Z represents the retardation introduced by the growth regulator; thus when no growth regulator is added $Z = 0$ and the equation becomes

$$\log_e \text{weight} = K + Bt.$$

Examples of the agreement between the observed and calculated values are given in Fig. 3. Inspection shows that it is possible to determine for each concentration the time elapsing before any selected level in the depression of the growth rate is reached. For comparative purposes a line having a slope equal to 75 per cent. of the control was drawn through the origin and at each concentration the time taken for the growth curve to intersect this line determined.

From Table II it is evident that the period elapsing before intersection differs greatly between temperatures, the interval decreasing as the temperature rises. It would also appear that when the effects of light intensity are less well defined, there is a trend for growth to be retarded less at the lowest level.

Net assimilation rate. Since the previous experiment had demonstrated that sodium 2:4-dichlorophenoxyacetate depressed the rate of dry matter production, the next logical step was to ascertain whether the depression in the relative growth rate was linked with a lowering of the net assimilation rate.

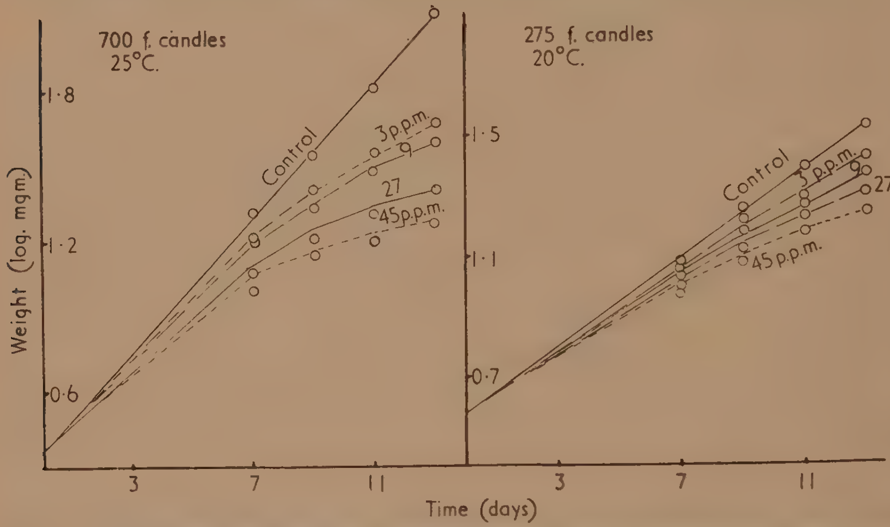

FIG. 3. The calculated curves and the observed values for the changes in weight (logarithmic scale) with time when the culture solution contains varying concentrations of sodium 2:4-dichlorophenoxyacetate and the plants are subjected to various combinations of light intensity and temperature.

Cultures of *L. minor* with and without the addition of a sublethal concentration of the growth regulator (27 p.p.m.) were grown under the same nine concentrations of light intensity and temperature, viz. 180, 275, and 700 foot-candles and 20°, 25°, and 30° C. Dry weight and area determinations were

TABLE II

The effects of light intensity and temperature on the time taken before the growth rate of L. minor, *treated with sodium 2:4-dichlorophenoxyacetate, is reduced to 75 per cent. of that of the corresponding control rate*

Light intensity (foot-candles)	Temperature (° C.)	Time (days) Concentration (p.p.m.)			
		3	9	27	45
700	30	10	8	7·5	5
	25	13	12	9	7·5
	20	—	14	11	8
275	30	7·5	7·5	7	4·5
	25	13·5	12	10	7
	20	—	—	13	11
180	30	9·5	7·5	7	7
	25	—	15	12	8
	20	—	—	14·5	11

made at the commencement of the experiment and at the end of each of two successive 2-day periods.

During the short experimental period there was little deviation from linearity between area and weight in either the control or treated series (see Fig. 4). Such small deviations as were apparent tended to increase with temperature and were greatest at the low light intensity and highest temperature. The magnitude of these deviations was considered to be such that the standard formula for net assimilation rate could be applied without involving large errors (Williams, 1946).

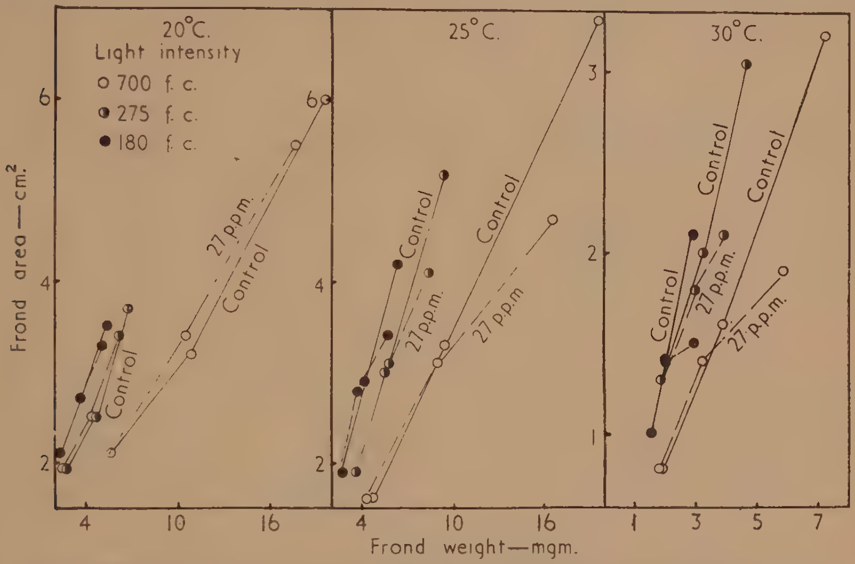

FIG. 4. The relationship between frond area and frond weight when the culture solution contains 27 p.p.m. of sodium 2:4-dichlorophenoxyacetate (broken lines) and the plants are subjected to different combinations of temperature (20°, 25°, and 30° C.) and light intensity (700, 275, and 180 foot-candles). Measurements of area and weight were made at 0, 2, and 4 days.

Statistical analysis of the data given in Table III shows that all four of the main factors—temperature, light intensity, concentration, and period—caused significant variations ($P = 0{\cdot}01$). At the same time there was a just significant interaction between temperature and light intensity, but there was no significant interaction between concentration and any of the other variables. Although, in the first period, the net assimilation rate of the treated plants under a combination of high temperature and high intensity was significantly greater than the control, there was a general tendency for the phenoxyacetic acid to reduce the net assimilation rate. However, only two of the mean values were significantly lower than the corresponding controls, these occurring at the highest temperature in the second period. Over all combinations of temperature and light intensity the mean depressions in the two periods were 2 and 12 per cent. respectively.

Induction of chlorosis. In the experiment relating to the depression of the relative growth rate it will be recalled that at a concentration of 45 p.p.m. a few of the fronds became chlorotic, and it seemed of interest to investigate how far the capacity of 2:4-dichlorophenoxyacetic acid to induce chlorosis was linked with variations in temperature and light intensity. The methods for assessing the degree of chlorosis and the statistical technique of probit analysis employed in the interpretation of the data have already been described (p. 159).

Pilot experiments, covering three light intensities (180, 275, and 700 foot-candles) and two temperatures (20° C. and 25° C.), were first carried out to determine the appropriate concentration range for each set of conditions. These pilot experiments indicated that the level of light intensity influenced the response such that the degree of chlorosis was greater at a low intensity. Following these experiments, a further, larger experiment was designed to include the effects of varying the light intensity both before and after the plants are placed in the solutions containing the growth regulator.

TABLE III

The interrelated effects of temperature, light intensity, and sodium 2:4-dichlorophenoxyacetate on the net assimilation rate of L. minor

Light intensity (foot-candles)	Conc. growth regulator in culture (p.p.m.)	Net assimilation rate (mg./cm.^{2}/day)					
		0–2 days			2–4 days		
		Temperature ° C.					
		30	25	20	30	25	20
700	0	0·85	1·04	0·99	0·76	1·04	0·98
	27	0·98	1·00	0·89	0·59	0·99	0·84
275	0	0·39	0·44	0·43	0·31	0·43	0·36
	27	0·36	0·39	0·42	0·26	0·38	0·30
180	0	0·21	0·33	0·27	0·25	0·31	0·25
	27	0·21	0·27	0·27	0·20	0·29	0·26

Significant difference between treatments ($P = 0·05$) = 0·12.

The procedure was the same at both temperatures, but limitations of space in the baths prevented a simultaneous comparison at both temperatures. At each temperature plants were grown at the three light intensities for a period of 14 days prior to treatment with the phenoxyacetic acid at several levels of concentration. Thus, in all there were three levels of 'pretreatment' light intensity combined with three levels of post-treatment and seven concentrations. Replication was obtained by two identical blocks (at each temperature) separated in time. In the pretreatment period for the second block, at 25° C., some difficulty was experienced in maintaining a constant light intensity since fluctuations in mains voltage due to 'load-shedding' were particularly great and on two occasions there were short power failures.

The number of fronds exhibiting the standard degree of chlorosis after 3 days was recorded for each culture, and this number was expressed as a percentage of the initial number of fronds: thus, the results in this form could be analysed satisfactorily by the probit method. Statistical analysis revealed that at 20° there was a considerable variation so that chi-square for heterogeneity was significantly large. However, for each of the three levels of intensity prior to treatment the six probit lines (three levels of post-treatment light intensity in each of two blocks) did not differ significantly among themselves either in slope or position; hence a single line was fitted for each intensity of the pretreatment period (Fig. 5).

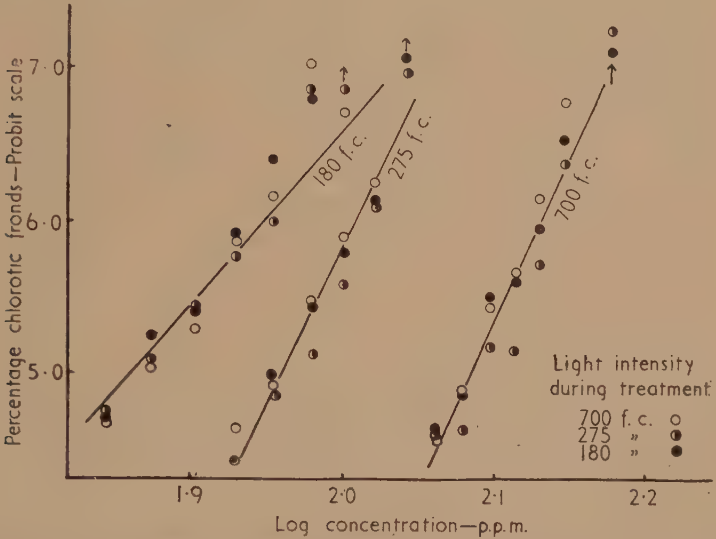

FIG. 5. The relationships between the degree of chlorosis induced by varying concentrations of sodium 2:4-dichlorophenoxyacetate and the intensity of the light received either before or during treatment. The straight lines are the fitted regressions (Table IV) linking chlorosis (probit scale) and concentration (log. scale) for each of the levels of intensity (700, 275, and 180 foot-candles) in the period *prior* to treatment.

The slopes of the three probit lines—see Table IV—for the pretreatment light intensities were significantly different ($P < 0{\cdot}001$): thus, it is not possible to evaluate the relative effects of the pretreatment intensities in terms of a single concentration which produces a standard degree of chlorosis. However, over the concentration range which for 20° C. extended from 70 to 150 p.p.m. the amount of chlorosis decreased as the intensity in the pretreatment period increased.

The results were more consistent at 25° C. since the heterogeneity chi-square values were not significantly large. There was no significant departure from parallelism shown when all eighteen lines were considered together, but there was a significant block difference. For each block and each pretreatment intensity the three lines for the different levels of post-treatment intensity did not differ significantly in position, so that single lines could be fitted for the pretreatment effects. On the other hand, as Table IV demonstrates, there was an extremely significant difference between lines for the three intensities prior to treatment, while the block effect also differed between intensities.

TABLE IV

The effect of varying the light intensity prior to the treatment on the chlorosis of L. minor *induced by sodium 2 : 4-dichlorophenoxyacetate at three levels of light intensity and at two temperatures.* (*Effects of pretreatment expressed as probit regression lines linking degree of chlorosis with concentration*)

Light intensity of pretreatment (foot-candles)	Probit regression line $y =$ 20° C. $[x = (\text{log. conc.}) - 1{\cdot}80]$		Probit regression line $y =$ 25° C. $[x = (\text{log. conc.}) - 1{\cdot}60]$
180	$11{\cdot}48x + 4{\cdot}29$	Block 1.	$14{\cdot}85x + 2{\cdot}71$
		2.	$14{\cdot}85x + 3{\cdot}14$
275	$20{\cdot}55x + 1{\cdot}71$	1.	$14{\cdot}85x + 2{\cdot}06$
		2.	$14{\cdot}85x + 2{\cdot}51$
700	$21{\cdot}65x - 1{\cdot}16$	1.	$14{\cdot}85x + 0{\cdot}46$
		2.	$14{\cdot}85x + 0{\cdot}63$

At 25° C. the influence of light intensity in the pretreatment period is similar to that at the lower temperature, that is, the concentration required to produce the same degree of chlorosis in the post-treatment phase is greatly decreased as the light intensity is reduced. Again, it would appear that during the short time the fronds are exposed to the action of the growth regulator, the effects are independent of light intensity over the range of 700—180 foot-candles.

In Table V the values for the equi-effective concentrations inducing 50 per cent. chlorosis have been calculated for each temperature and each intensity

TABLE V

The effects of varying temperature and light intensity on the equi-effective concentration of sodium 2 : 4-dichlorophenoxyacetate required to produce chlorosis in half the fronds of L. minor

Temperature (° C.)		Light intensity of pretreatment (foot-candles)	Equi-effec. conc. (p.p.m.)	Upper and lower fiducial limits of L.D. 50 ($P = 0{\cdot}05$)
20		180	73	71–75
		275	91	90–92
		700	121	120–122
25	Block 1.	180	57	54–59
	2.	,,	53	52–54
	1.	275	63	60–66
	2.	,,	59	58–60
	1.	700	81	79–82
	2.	,,	79	77–80

in the pretreatment period. Since these values have been derived from lines which have significantly different slopes, it is not possible to combine the results for both temperatures in a single analysis. However, it is evident that at each intensity the physiological action of the growth regulator is greater at the higher temperature.

It is difficult at 25° C. to account for the block difference, but it is possible that the greater effect in the second block resulted from the reduced light intensity associated with the voltage fluctuations of the electricity supply.

Inspection of Fig. 4 demonstrates that irrespective of whether the plants are treated with sodium 2:4-dichlorophenoxyacetate the ratio of frond area to weight is increased as the light intensity is reduced. Other observations showed that a reduction in light intensity also produced shorter roots. Thus, at the end of the pretreatment period the ratio of surface to weight in contact with the solution and the length of the roots would be dependent on the intensity. It could therefore be postulated that the lower concentration needed to induce chlorosis subsequent to exposure to the lowest intensity was linked with a greater absorption of the growth regulator because of the relative gain in surface area. Such an hypothesis would carry the corollary that absorption through the under surface of the frond is more important than uptake by the roots.

Gorham (1941) found that salt uptake took place largely via the under surface of the frond, but the possibility that this did not hold for 2:4-dichlorophenoxyacetic acid was investigated. A series of beakers, each containing fifty fronds, were taken, and in half the roots were cut off approximately 2 mm. from the fronds, while a liberal smear of lanolin paste was applied to the underneath surface of the fronds in half the beakers in each group. The plants in all beakers were exposed to a concentration of 140 p.p.m. for 24 hours; the fronds were then washed and transferred to the standard culture solution for 2 days. The number of fronds exhibiting severe chlorosis in each beaker was then recorded. From Table VI it is clear that all the fronds that had not been treated with lanolin were injured while most of the remainder were but slightly affected. These results indicated that at least a considerable proportion of the absorption was through the under surface of the frond. Moreover, the number of fronds treated with lanolin which were chlorotic was independent of the status of the roots.

TABLE VI

The effects of root removal and the placement of lanolin on the lower surface of fronds of L. minor *on the percentage of chlorotic fronds produced by sodium 2:4-dichlorophenoxyacetate*

	Percentage of chlorotic fronds	
	Without lanolin	With lanolin
Roots present . . .	100	6
Roots absent . . .	100	8

Another approach was to compare the changes in dry weight of normal fronds with others in which the roots had been removed. Initial dry-weight determinations were made on samples from both groups, while other samples were treated for 24 hours with a 50-p.p.m. solution of the growth regulator. Subsequently all cultures were grown for a further 7 days in the normal solution before they were harvested. The increases in the logarithms of dry weight were recorded and have been expressed in Table VII as percentages of the controls with intact roots. The gain in weight was not reduced by the temporary removal of the roots, nor did this operation have any significant effect on the inhibition of growth by the phenoxyacetic acid. Thus these experiments confirm the findings of Gorham (1941) that uptake is largely through the under surface.

TABLE VII

The effect of removing the roots on the response of L. minor *to sodium 2:4-dichlorophenoxyacetate*

	Logarithmic increase in dry weight (% of control) Concentration (p.p.m.) 0	50
Roots present . . .	100	81
Roots removed . . .	99	83

Significant difference (*P* = 0·05) 7.

To substantiate that the amount of growth regulator absorbed subsequent to pretreatment is linked with the surface area proved difficult to test experimentally, for it was not possible to vary the ratio of area to weight without altering the physiological status of the fronds and thus possibly the reaction to the growth regulator. If the hypothesis is restricted to the contention that for a given concentration uptake is solely proportional to the surface area, then the equi-effective concentration should be inversely proportional to the

TABLE VIII

The interrelated effects of temperature and light intensity on the ratio of area to weight of L. minor *and the equi-effective concentration of sodium 2:4-dichlorophenoxyacetate required to induce chlorosis*

Temp. (° C.)	Light intensity (foot-candles)	Ratio of area to weight (cm.^{2}/mg.)	Equi-effect. conc. (p.p.m.)	Product of ratio and equi-effect. conc.
20	180	0·48	73	35·0
	275	0·41	91	37·3
	700	0·27	121	32·6
25	180	0·69	55	38·0
	275	0·61	61	34·1
	700	0·35	80	28·0

area: weight ratio, that is, the product is a constant. To test this postulate, the ratios of area to weight for the three sampling occasions of Fig. 4 were obtained for the corresponding light intensities of the present experiment and the mean values multiplied by the equi-effective of concentrations. From Table VIII it is apparent that the resultant products were not constant, particularly at 25° C. It must therefore be concluded that the influence of light intensity prior to exposure to the growth regulator cannot solely operate through changing the ratio of area to weight.

DISCUSSION

It has been established that irrespective of the levels of temperature and light intensity there is a cumulative depression in the growth rate at sublethal concentrations of the growth regulator. Such a cumulative response could operate through *(a)* progressive falling off in photosynthetic activity, *(b)* a progressive diminution in the proportion of assimilatory tissue, and *(c)* a rising respiration rate. Ashby and Wangermann (1951) have shown that the growth rate of a frond is sigmoid and any inhibition of the rate of formation of new fronds would lead to a population with a changing proportion of senescent fronds, and, in consequence, a possible changing balance between photosynthetic activity and respiration. It has also been found that 2:4-dichlorophenoxyacetic acid causes with time morphological deformities, such that the upper surface of the frond becomes roughened and humped (Blackman and Robertson-Cuninghame, 1954). Such a change might affect the proportion of incident light penetrating the tissues, and it will be recalled that in measuring frond area only the outlines were recorded and thus no distinction is made between a curved and flat surface.

In view of these complexities, it is not possible to assess precisely the underlying causes leading to the small mean depression in the net assimilation rate – that is, the net balance between assimilation and respiration. Since over several of the combinations of light intensity and temperature no depression in the net assimilation was recorded (Table II), the direct effect of the dichlorophenoxyacetic acid on photosynthesis can have only been small under conditions where the plants exhibited little injury other than slight epinastic and morphological effects. In this connexion, Freeland (1949) after spraying beans with a 0·01 per cent. solution found that carbon dioxide fixation by the leaves was decreased, while the respiration rate after first being depressed was later increased. More recently Loustalot and Muzik (1953) working with *Stilozolobium deeringianum* have observed that spray concentrations which were eventually lethal (0·1 – 0·01 per cent.) caused within a short period marked reductions in photosynthesis but that at a concentration of 0·001 per cent. no depression was recorded until a week or more after treatment, when terminal growth had ceased and the stem exhibited marked morphological abnormalities. Lastly, Wedding, Erickson, and Brannaman (1954) investigated the effects of the same growth regulator on the photosynthesis of *(a)* submerged vacuum infiltrated disks punched out of orange leaves, and *(b)* aseptic cultures of

Chlorella pyrenoidosa. When the pH was maintained constant at pH 4·5 it was observed for both species that no marked depression in photosynthesis took place until the concentration reached the high figure of $4-6\times10^{-4}$ M, but to what extent the various concentrations depressed the relative growth rate of *Chlorella* was not recorded. By altering the pH an attempt was made to assess the importance of the degree of dissociation, but on the basis of the findings of Blackman and Robertson-Cuninghame (1953) the design of the experiments was not precise enough for critical conclusions to be reached.

Inspection of Fig. 3 shows that at a time when assimilation was relatively unaffected the total frond area was being reduced and the relative growth rate depressed. These observations, together with those of Loustalot and Muzik (1953), indicate that the growth-inhibiting action of 2:4-dichlorophenoxyacetic acid cannot primarily operate by interfering with assimilation. Rather, it would appear that the depressions in photosynthesis when they occur arise as the result of other effects. For example, Loustalot and Muzik (1953) have put forward the suggestions that abnormal developments in the conducting tissues of the stem and petiole might be contributory factors by causing both an accumulation of carbohydrates in the leaves and a restriction of the water supply and thereby a lowering of the assimilation rate. For *L. minor*, a relatively simple organism floating on water, it would seem unlikely that these factors can be of importance, since the two significant reductions in net assimilation rate occurred at the highest light intensity and the maximal levels of assimilation.

Irrespective of whether the response is the production of chlorotic fronds or a decrease in the rate of growth, the results demonstrate that the physiological action of 2:4-dichlorophenoxyacetic acid increases as the temperature rises. Since the growth of the controls is linked with both light intensity and temperature and since the depression in the growth rate is cumulative, a precise evaluation of the temperature coefficient cannot be made. On the basis of the time taken for the growth rate, relative to the control, to be reduced by 25 per cent. (Table II), the ratio of the periods taken at 20° C. and 30° C. lies within the range of 1·5 to 2·3. It must, however, be emphasized that this criterion of growth depression is not a strict measure of the rate of response, and the ratio of 1·5 to 2·3 is only indicative of the scale of Q_{10}.

There is the same difficulty of quantitative appraisal for the effects of temperature on the induction of chlorosis. Because the slopes of the probit lines differ between temperatures (Table IV), comparative figures of equi-effective concentrations at the two temperatures cannot be obtained. However, the present data confirm on a more quantitative basis the conclusions reached by Kelly (1949) that the toxicity of 2:4-dichlorophenoxyacetic acid is positively linked with temperature.

The effects of light intensity are clearly complex, and there is some divergency of trend. In terms of the induction of chlorosis, the lower the light intensity prior to treatment the smaller is the concentration required to bring about the standard response (Table V). On the other hand, when the data for adjusted logarithmic growth are considered, over all concentrations the depression tends to be greater at the highest light intensity (Figs. 1 and 2). It has already been pointed out that this adjusted scale allows of a comparison of the proportional reduction in growth under the different levels of light intensity and temperature. However, for a given time interval the analysis of the data does not take into account that, due to varying growth rates, a different number of frond generations will have been produced under the several combinations of light and temperature and that the over-all effect of the growth regulator may be linked with the rate of frond multiplication.

TABLE IX

The effects of varying light intensity and temperature on the time taken for control cultures to double their weight

Light intensity (foot-candles)	Time (days) Temperature 30° C.	25° C.	20° C.
700	2·01	2·23	2·77
275	3·40	2·89	4·14
180	4·88	4·14	5·15

From the regressions of Table I it is possible to calculate the time taken for the weight to be doubled in the control cultures, and this will reflect a corresponding change in frond number. Since the interval is inversely proportional to the regression coefficient, it follows that the interval increases as the light intensity decreases. From Table IX it is evident that for a combination of 700 foot-candles and 30° C. the interval is less than half that at 180 foot-candles and 20° C. In consequence, at each temperature the number of generations exposed to the growth regulator is greatest at the highest level of light. Therefore, provided that there is no direct effect of light intensity, a larger response could be postulated at 700 foot candles merely because the multiplication rate is faster. On the other hand, it has been found that though between intensities the interval is very different, the order of depression induced by the growth regulator only varies a little with intensity. These facts suggest that the effect per generation is most marked at the lower intensities.

Further verification of this conclusion has been obtained by first calculating the time taken for the control at each combination of temperature and intensity to quadruple the initial weight and then to determine from the series of growth curves (p. 163) the actual change in weight of the treated cultures over the corresponding interval for the appropriate control. Inspection of Table X shows that the relative inhibition of growth is increased as the light intensity is reduced. It is also of interest to note that the conclusions concerning the temperature factor remain unaltered: the response is still proportional to temperature.

In considering ways in which temperature may operate, the conclusion reached by Weintraub and Brown (1950*b*) that temperature primarily increased the translocation of the dichlorophenoxyacetic acid in beans is hardly applicable to *L. minor* where the whole of the under surface was in contact with the culture medium and the paths of transport to all parts of the tissue very short. The more likely explanations are that temperature increases the rate of uptake or accelerates the physiological effects at cell level. In this laboratory Reinhold (1954) has shown that the uptake of indolylacetic acid by pea epicotyls and carrot disks is controlled by two groups of processes, one metabolic and the other physical, and that the temperature coefficient for the metabolic system is approximately 2·6. If similar mechanisms govern the absorption of 2:4-dichlorophenoxyacetic acid, then an increased inhibition of growth with rising temperature would be expected. To substantiate this hypothesis attempts were made to measure the changes in the optical density of the culture solutions at 2,300 Å. with a spectrophotometer, but the method was not sensitive enough to detect the very small quantities absorbed. For example, when 200 fronds, weighing 187 mg., were placed in 100 ml. of solution for 48 hours the change in the content of the solution was less than the amount which could be estimated with accuracy, namely, 0·2 mg. From Table VIII it has been seen that the interrelationship between the concentration required to produce chlorosis and the light intensity prior to treatment cannot be fully explained on the basis that the amount of growth regulator absorbed is dependent on the combination of the external concentration and the ratio of frond area to frond weight. It might be advanced that as a result of the reduction in intensity the absorption of the growth regulator was increased, but the limited evidence at present available suggests that the salt uptake of intact plants is decreased by shading, e.g. Blackman and Rutter (1949).

Table X

The calculated gain in weight of cultures treated with sodium 2:4-dichlorophenoxyacetate over a period equivalent to the time taken for control cultures to quadruple their weight at each combination of light intensity and temperature

Conc. growth regulator (p.p.m.)	Increase in weight—log. scale								
	30° C.			25° C.			20° C.		
	700	275	180	700	275	180	700	275	180
	(foot-candles)			(foot-candles)			(foot-candles)		
0	0·60	0·60	0·60	0·60	0·60	0·60	0·60	0·60	0·60
3	0·55	0·48	0·44	0·58	0·55	0·54	0·58	0·56	0·56
9	0·51	0·47	0·41	0·56	0·55	0·52	0·57	0·53	0·54
27	0·50	0·45	0·39	0·55	0·52	0·49	0·54	0·52	0·50
45	0·50	0·37	0·37	0·51	0·47	0·44	0·50	0·49	0·46

A number of investigators have observed that following on treatment with 2:4-dichlorophenoxyacetic acid there is a rapid disappearance of carbohydrate reserves and that this loss, though not a primary factor leading to the death of the tissues, might still be of some importance. Accordingly, at the end of the experimental period fronds from each treatment were examined for the presence of starch and it was found that irrespective of the light intensity during or before treatment the chlorotic fronds contained some starch. These observations do not rule out the possibility that the level of carbohydrates may have some significance, but it is evident that the chlorotic symptoms can take place when starch is still present in the tissues.

Turning to a consideration of other internal factors that may be involved, Wangermann and Ashby (1950 and 1951) have concluded that the area of a frond is controlled by some stimulatory substance produced by the parent frond and that late-formed daughter fronds are small because the amount or activity of the stimulatory substance received from the parent is low. This reduction in area of the daughter fronds could not be counteracted by the addition of 2·5 p.p.m. of indolylacetic acid to the culture solution, but Blackman and Robertson-Cuninghame (1954) have observed that the over-all growth rate of a population of *L. minor* on either a weight or an area basis can be increased by concentrations up to 25 p.p.m. They also observed that in the presence of indolylacetic acid the relative inhibition of growth brought about by sodium 2:4-dichlorophenoxyacetate was reduced, and the conclusion was reached that some of the observed interactions could be interpreted by assuming that the two compounds act competitively on a common system. This postulate could be extended if it is further assumed that the light factor can affect the balance of such a system. Thus, if the content of indolylacetic acid is inversely related to the light intensity it would be expected that the effects of the dichlorophenoxyacetic acid would be accentuated at low intensities. On the other hand, it has been emphasized (Blackman and Wilson, 1953) that the mechanisms and factors governing the photoinactivation of indolylacetic acid are still obscure, while the role of other natural growth substances and growth inhibitors has yet to be elucidated.

In conclusion, it is clear that without further investigation it is not possible to differentiate between the effects of light and temperature on the uptake of the growth regulator and the physiological changes induced in the tissues. On the available evidence it seems likely that both uptake and the effects at cell level are linked with temperature, while the light factor influences the internal changes more than absorption. Evidence in support of the second conclusion is forthcoming from complementary experiments on *Helianthus annuus* (Blackman and Robertson-Cuninghame, 1955).

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Interrelationships between Light Intensity and the Physiological Effects of 2:4-Dichlorophenoxyacetic Acid on the Growth of *Helianthus annuus*

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SUMMARY

The interrelationships between light intensity and the effects of sodium 2:4-dichlorophenoxyacetate on the growth of *Helianthus annuus* in the early vegetative stage have been studied by subjecting treated and control plants to a range of light intensities from 1·0 to 0·12 daylight. The growth regulator in varying amounts was applied either as droplets of aqueous solution to the first or second pairs of leaves or as an overall spray. When the amount of the compound is such as to cause small but significant reductions in the relative growth rate, the leaf-area ratio and the ratio of leaf area to leaf weight are likewise depressed but the net assimilation rate is relatively unaffected. Between 0·25 daylight and full daylight the proportionate changes in the relative growth rate, leaf-area ratio, and net assimilation rate induced by sub-lethal amounts of the growth regulator are not greatly modified by the level of light. If the intensity is reduced further to 0·12 daylight, then the reactions of the shaded plants differ markedly from those of unshaded plants. For example, when the plants are shaded both before and after a spray application, the concentration required to cause a 50 per cent. mortality is one-tenth of that demanded for plants receiving full daylight. For less phytotoxic amounts the percentage reductions, relative to the controls, induced in the growth rate of the shoot are more dependent on the intensity after than before the application of either a spray or measured droplets.

These greater depressions in shoot growth at 0·12 daylight after the application are linked with comparable depressions in the rate of growth of the leaves of which the first pair are more sensitive. Similarly, the growth rate of the first internode is also depressed more under shade conditions, but that of the second internode at low doses may be greatly increased. For both the leaves and internodes shading before as against shading after the application may have different significant effects on the changes in growth caused by the growth regulator. Under some conditions the interactions between the light intensity before and after the application and the quantity of the growth regulator are exceedingly complex. By using sodium 2:4-dichloro-5-iodophenoxyacetate, labelled with radioactive I^{131}, it has also been established that the rate of penetration into leaves is accelerated by a high light intensity after the application, but that the intensity received prior to the application causes no significant effect. Evidence was also obtained that transport to the shoot from the treated leaf is more dependent on the light intensity after the application—the transport being greater in full daylight. It is concluded that a number of factors must be involved in bringing about these differential effects and their relative importance is discussed.

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INTRODUCTION

PREVIOUS investigations relating to the influence of light on the physiological activity of 2:4-dichlorophenoxyacetic acid have already been reviewed in a recent paper (Blackman and Robertson-Cuninghame, 1955) which was concerned with a study of the changes in the growth rate and development of *Lemna minor* induced by 2:4-dichlorophenoxyacetic acid under conditions where light intensity and temperature could be maintained at varying but constant levels. It was found that when the concentrations in the culture solution were low, then the power to produce a small but standard retardation in the growth rate was greatly enhanced by rising temperature while the growth of plants receiving the highest light intensity was slightly more inhibited. At higher concentrations which led to the destruction of the chlorophyll within 72 hours, the concentration required to cause a standard degree of chlorosis fell as the temperature rose. Varying the light intensity during the period of treatment was without effect. On the other hand, the lower the intensity under which the plants were grown prior to treatment the smaller was the subsequent concentration needed to induce chlorosis.

In the present and to some extent complementary investigation an examination has been made of the effects of shading on the changes in the net assimilation rate and vegetative growth of *Helianthus annuus* induced by the same growth regulator. The general plan of the main series of investigations was to sow seeds in pots in the open and to start the experiments when the third pair of true leaves was clearly visible. The plants in this early vegetative phase, either before or after treatment with the growth regulator, were either left uncovered or placed under perforated metal screens with transmission factors of 0·5, 0·25, and 0·12. Sodium 2:4-dichlorophenoxyacetate, in aqueous solution, was applied (*a*) to the shoot as an over-all spray, or (*b*) to the leaves as measured droplets from a micro-pipette.

During the experimental period samples of pots were withdrawn from time to time and some or all of the following determinations made on each occasion: the dry weights of the root, stem (divided into hypocotyl and first and second internodes), leaves (divided into first pair and younger leaves), while the leaf areas were also estimated. The resultant data were subjected to the methods of growth analysis which have been previously employed to assess the effects of light intensity on the net assimilation rate and the relative growth rate of the sunflower and its parts—e.g. Blackman and Wilson (1951 and 1954).

In an ancillary series of experiments which related to the influence of light on the rate of penetration into leaves and the subsequent transport in the plant 2:4-dichloro-5-iodophenoxyacetic acid containing radioactive I^{131} was used.

EXPERIMENTAL METHODS

Since most of the experimental techniques and methods have been fully described in recent papers (Simon, Roberts, and Blackman, 1952; Blackman and Wilson, 1954; Blackman and Robertson-Cuninghame, 1954) only a summary will be given except for those aspects which have not yet been published.

Seeds of the variety 'Pole Star' were sown in a sand-soil mixture in glazed earthenware or aluminium pots of 10-in. diameter and the ultimate number of plants reduced to 5–7 per pot. At the start of the experiment the pots were arranged in the appropriate blocks and pots selected at random for the initial and subsequent samplings. On each occasion the shoot was cut off at ground level and immediately divided into hypocotyl, first internode, second internode, first pair of leaves, and second pair of leaves which included the apex. These parts, together with the roots, were dried separately at 100° C. and weighed. In the largest experiments where time and labour did not permit of the recovery of the roots, the shoot was cut off at the cotyledonary node; otherwise the same procedure was followed. To collect the roots, the soil from each pot was carefully washed through a sieve on which the roots were retained.

For the estimation of the leaf areas, a subsample of leaves from each treatment was placed between sheets of glass on blue print paper before they were dried and weighed separately. The areas on the blue prints were subsequently determined by a planimeter.

On the night prior to the application of the growth regulator the pots were transferred to the greenhouse and kept there until 18 to 24 hours after treatment. Save for the experiments involving radioactive tracers, they were then placed in the open.

The spray apparatus in the greenhouse was designed to supply a reproducible spray pattern. The replicate pots for each treatment were set up in line beside a track along which the apparatus with a single fan jet nozzle could be moved at a variable but constant speed. By means of a compressed air line and a control system the pressure inside the glass container holding the spray solution could be maintained at a constant pressure so that the output through the nozzle remained uniform. In the present experiments the pressure was standardized at 47 pounds per square inch, and with a Monarch No. 59 nozzle the output was equivalent to approximately 120 gallons per acre.

For the droplet application the measured drops were placed on the leaves by means of an 'Agla' micrometer syringe which it is claimed will deliver droplets of 0·001 ml. with an error of ±0·00005 ml. When the growth regulator contained radioactive material the syringe was enclosed in a specially constructed lead cylinder to which a metal handle was attached.

The screens employed for reducing the light intensity consisted of light wooden frames (usually 6×4 ft.) covered with perforated zinc and with side pieces of the same metal. Each frame was suspended over a group of pots and the height could be adjusted as the plants grew. Normally the bottom of the sidepieces were at the same level as the top of the pots, so that while the plants did not receive additional lateral illumination there was free circulation of air. Comparison of thermograph records under the screens with those of a thermograph in a standard Stevenson screen confirmed previous experience that the mean daily air temperature was unaffected by this shading technique. In the greenhouse the same type of screen was employed but the dimensions were altered to fit the benches.

In certain of the experiments where the plants had been grown under different light intensities prior to the spray application, it was necessary to determine how far the morphological changes due to shading affected the quantity of spray solution retained by the shoot. Additional groups of pots were therefore included in the experiment for this purpose and were sprayed with a 1 per cent. solution of the yellow dye tartrazine which it had been found previously was only very slowly absorbed by the shoot. After allowing the excess spray to 'run off', the plants in each pot were cut off at ground level and the adhering dye washed off with distilled water into a beaker. Each washing was made up to a standard volume and the optical density measured with a 'Spekker' photo-electric absorptiometer. At the same time a calibration curve linking optical density and concentration was constructed and by reference to this curve the volume of spray retained could be calculated. It should be pointed out that the surface tension of the tartrazine solution was somewhat lower than those of the growth regulator, so that the relative rather than the absolute differences in retention were estimated.

When sodium 2:4-dichloro-5-iodo^{131} phenoxyacetate was applied as droplets to the leaves, in order to recover the active absorbed material the various tissues were first macerated in alcohol either by grinding small portions in specially designed glass grinders or, for the more bulky tissue, in a mechanical 'blender'. Each sample was then made up to a convenient minimum volume with alcohol and the plant material deposited as a pad by centrifuging. Following the technique of Martin and Russell (1950), the activity of a 10-ml. aliquot of the supernatant liquid was determined using liquid-assay Geiger-Muller counters. Further details are given in the text.

EXPERIMENTAL RESULTS

I. *The interrelationship between light intensity and the effects of sodium 2:4-dichlorophenoxyacetate on growth and vegetative development*

The first full-scale experiment aimed at determining the effects of varying light intensity on growth after a uniform population of young plants had been sprayed with a solution containing 40 p.p.m. of sodium 2:4-dichlorophenoxyacetate. To ensure that the absorption was of the same order, after spraying all plants were kept in the greenhouse for 18 hours, and then the shoots were thoroughly washed with water to remove any spray residues. After this treatment the pots were placed in the open and subjected to three levels of light intensity, namely, 1·0, 0·5, and 0·25 daylight. At the time of the initial sample, the leaves of the first pair were 4 to 5 cm. long. Further samples were taken at the end of 6 and 12 days.

The first analysis of the data was to determine whether a linear relationship existed between plant weight and leaf area over the whole experimental period, and examination showed that there was considerable deviation from linearity.

This deviation occurred with all treatments and is ascribed to the greater growth in the second period due to the more favourable environmental conditions. Using the integrating light recorder previously described (Blackman, Black, and Martin, 1953) the mean light received per day was 23,000 foot-candle hours in the first 6 days and 35,000 in the next period, while the mean daily temperature was also higher—18 as against 16° C. It was not therefore valid to use the formula for net assimilation rate over the whole 12-day period, but it did not seem likely that large errors would be introduced if the formula was employed for each of the 6-day periods.

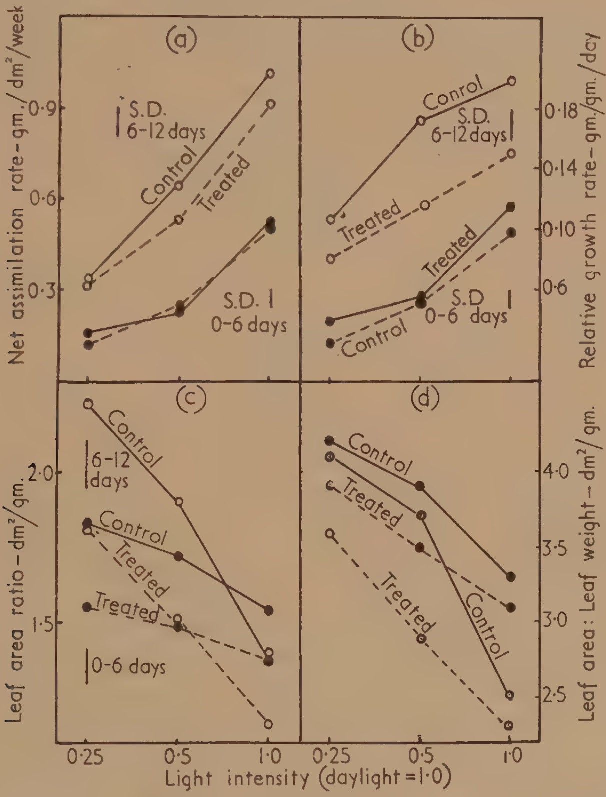

FIG. 1. Expt. 1. The effects of a spray application of 40 p.p.m. of sodium 2:4-dichlorophenoxyacetate on (*a*) the net assimilation rate, (*b*) the relative growth rate, (*c*) the leaf-area ratio, and (*d*) the ratio of leaf area to leaf weight of *H. annuus*, grown under three light intensities subsequent to spraying. The changes are recorded for the periods 0–6 days (—●—) and 6–12 days (—○—).

From Fig. 1*a* it is evident that the growth regulator caused no significant change in net assimilation rate during the first period but brought about a small but significant ($P = 0·01$) decrease during the second period, while the depression was slightly greater at the middle light intensity. Similarly the relative growth rate (Fig. 1*b*) was significantly depressed in both periods, the degree of depression again being greater in the second period. In this instance the order of the reduction was greater in full daylight than at 0·25 daylight.

Since the phenoxyacetic acid caused a considerable change in the relative growth rate but induced little change in the net assimilation rate, it follows that there must have been a considerable change in the leaf-area ratios. When these ratios were analysed statistically, highly significant differences were found between concentrations and intensities—see Fig. 1*c*. In both periods plants treated with the growth regulator were less leafy at all light intensities.

Although the actual amount by which the growth rate was depressed differed at different light intensities, the interpretation of any interaction with light intensity is complicated by the fact that the control growth rates were also greatly influenced by the level of light. In order to put the results on a relative basis the various rates and ratios have been expressed as percentages of the corresponding control figures at each light intensity. From Table I it is evident that, with few exceptions, the depressions were greater in the second period, but there was no marked interaction with light intensity.

TABLE I

Expt. 1. The effects of varying light intensity on the relative depressions caused by sodium 2:4-dichlorophenoxyacetate

	% of control					
	0–6 days			6–12 days		
	1	0·5 daylight	0·25	1	0·5 daylight	0·25
Relative growth rate . . .	86	93	67	76	67	75
Net assimilation rate . . .	96	106	76	90	83	94
Leaf-area ratio	91	87	86	85	80	81
Leaf area:leaf weight ratio . .	94	90	93	92	78	88

From the investigations of Blackman and Wilson (1953) it was known that the ratio of leaf area to leaf weight and the proportions of leaf stem and root are greatly altered by shading. Accordingly, the induced changes in this ratio together with the changes in the percentage of the total dry weight comprised by the roots, stems, and leaves were calculated. From Fig. 1*d* it is apparent that the mean leaf area: leaf weight ratio is depressed by the phenoxyacetic acid but the effect of light intensity is similar in both the control and treated plants. Similarly, from Table II it is clear that at all light intensities the growth regulator has reduced the proportion of leaves but has tended to increase the proportion of stem. Irrespective of the growth regulator effects, as the light intensity is reduced the proportion of stem increases at the expense of the root while there is but a slight effect on the proportion of leaves.

In Expt. 1 it had been observed that signs of chlorosis were almost entirely confined to the bases of the young leaves. However, since these leaves formed a cup in which spray droplets could collect, it was not known whether the greater chlorosis was due to a localized retention of the spray, a localized accumulation through translocation, or a localized sensitivity of the tissues. In addition, the failure of the light factor to interact with the effects of the phenoxyacetic acid might be due to the fact that the presence of the growth regulator in any part of the shoot did not necessarily depend upon translocation over any considerable distance. The next step was to investigate the effects of shading after the growth regulator, in droplet form, had been applied to particular points on the plant.

TABLE II

Expt. 1. The effects of varying light intensity and sodium 2:4-dichlorophenoxyacetate on the proportion of leaf, stem, and root

	Proportion (% of total dry weight)						
	Control			Treated			
	1	0·5	0·25	1	0·5	0·25	Sig. diff.
0–6 days		daylight			daylight		$(P = 0\cdot35)$
Leaf	57	58	60	49	51	51	3
Stem	19	23	23	27	29	29	3
Root	25	20	17	23	21	19	4
6–12 days							
Leaf	44	47	49	40	46	47	3
Stem	26	33	35	28	33	33	3
Root	30	21	16	32	21	20	4

Apart from the controls, the individual plants received two droplets each of 0·005 ml. of 0·1 per cent. aqueous solution of sodium 2:4-dichlorophenoxyacetate, that is 10 μg. per plant. In the first group one droplet was applied to the midrib of each of the first pair of leaves[1] at a distance of 1 cm. from the tip: in the second one droplet was placed on the midrib, near the base of the lamina, of each of the second pair. As in Expt. 1 the pots remained in the greenhouse for the first 18 hours, the spray residues were washed off, and the pots placed under the appropriate screens (1·0, 0·5, and 0·25 daylight). Ten days later, because of the labour involved, only four blocks were harvested, the remaining three blocks being harvested on the eleventh day.

Statistical analysis showed that the position of the droplet was of little significance; see Fig. 2. There is no significant effect of the growth regulator on net assimilation rate, but for both relative growth rate and the leaf area:leaf weight ratio the effects are highly significant $(P < 0\cdot001)$. The actual reduction in growth is least at 0·5 daylight, while the percentage reduction is very slightly greater at 0·25 daylight; see Table III. Similarly, the ratio of leaf area to leaf weight is also reduced, but, under conditions of shade, the reduction is just significantly greater when the phenoxyacetic acid is placed on the first leaf. On a percentage basis (Table III) there is little difference between light intensities.

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[1] The leaves are always numbered in a vertical series from above the cotyledons.

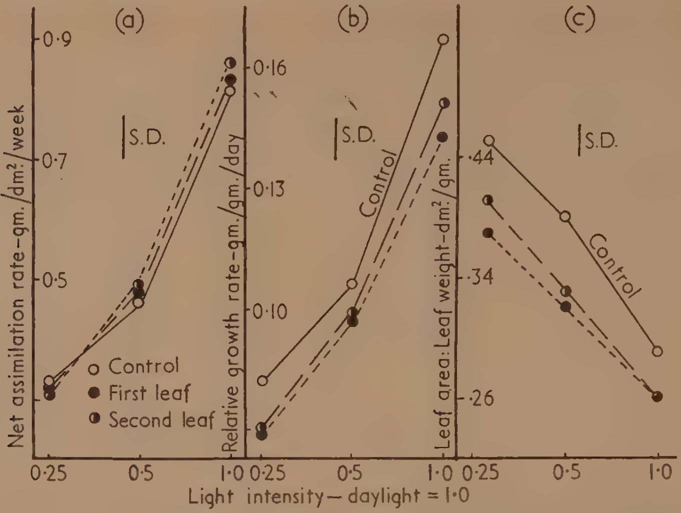

FIG. 2. Expt. 2. The influence of light intensity on the response of *H. annuus* to an application of 10 μg. of sodium 2:4-dichlorophenoxyacetate to either the first or second pair of leaves. The response measured in terms of (*a*) net assimilation rate, (*b*) relative growth rate, and (*c*) the ratio of leaf area to leaf weight.

TABLE III

Expt. 2. The effects of light intensity and the position of application of droplets of sodium 2:4-dichlorophenoxyacetate upon the relative growth rate and the leaf area : leaf weight ratio. Results expressed relative to the controls

	Percentage of control at each light intensity Position of droplets					
	1st leaf			2nd leaf		
	1·0	0·5 daylight	0·25	1·0	0·5 daylight	0·25
Relative growth rate (g./g./day) . .	86	92	84	90	92	85
Leaf area:leaf weight (Dm.²/g.) . .	88	86	87	89	87	90

From Table IV it is evident that the growth regulator brought about a significant increase in the proportion of stems at all light intensities while the roots were also significantly increased at the highest light intensity: at the same time at all levels of light the proportion of leaf was reduced. The effects were independent of whether the droplets were applied to the first or second pair of leaves.

A similar experiment, but including a screen transmitting 12 per cent. of daylight, was carried out later in the season. The plants at the time of the initial sampling were slightly smaller than those of the previous experiment since the leaves of the first pair were 3 to 4 cm. long. Again the amount of growth regulator applied was 10 μg. per plant, the droplets being placed on either the first or the second pair of leaves. Because of the size of the experiment—78 pots—both the initial and final sampling were spread over 2-day periods.

TABLE IV

Expt. 2. The effects of light intensity and the position of the application of droplets of sodium 2:4-dichlorophenoxyacetate on the proportion of leaf, stem, and root

Proportion (% of total dry weight)

Position of droplets

	Control			1st leaf			2nd leaf			
	1·0	0·5	0·25	1·0	0·5	0·25	1·0	0·5	0·25	Sig. diff.
	daylight			daylight			daylight			($P = 0{\cdot}05$)
Leaf	51·4	49·7	50·3	43·9	46·0	45·3	45·1	45·6	45·3	2·3
Stem	23·0	30·0	32·6	27·3	35·1	36·3	26·1	33·3	36·0	1·9
Root	25·4	19·0	17·6	29·1	19·0	18·1	28·9	21·3	17·3	2·4

Since the experiment was commenced on 19 September when the mean daily light intensity was low, both the relative growth rates and the net assimilation rate were also low. At 0·12 daylight the gain in weight of the control plants was negligible and they were considerably etiolated. The initial formative symptoms were similar at all light intensities and the young leaves showed severe basal chlorosis. Towards the end of the experimental period (14 days) the hypocotyls of treated plants at the lowest intensity became soft and collapsed. It was evident that death of these plants would have occurred had the experimental period been prolonged. The plants at the higher intensities were also severely affected but gave no indication that they would shortly die.

For the relative growth rates of (*a*) total plant weight, (*b*) stem weight, and (*c*) the area of the second pair and later leaves (young leaves) together with the net assimilation rate and the leaf area: leaf weight ratio, there were very highly significant differences between light intensities. The growth regulator caused significant reductions in the relative growth rates of total weight ($P < 0{\cdot}001$) and young leaves ($P < 0{\cdot}001$), significant gains in the stems ($P < 0{\cdot}05$), but no over-all significant decrease in the net assimilation rate (see Fig. 3*a*).

The relative growth rate of the whole plant (Fig. 3*b*) was depressed to the same extent by the applications to either the first or the second pair of leaves. The relative growth rate of the control plants at the lowest light intensity was negligible (0·03 g./g./day), while the corresponding rates for the treated plants were negative. Thus, when the results are expressed as a percentage of the controls at 0·12 daylight the values of the percentage relative growth rate of the treated plants are of the order of minus 100 per cent. (Fig. 4*a*).

The relative growth rate of the young leaves (Fig. 3*c*) was equally reduced by either application at all intensities. Thus, when these rates are expressed as percentages of the controls, as in Fig. 4*b*, there is no significant difference for sites of application but the percentage depression is just significantly increased by shading. On the other hand, the growth regulator caused an over-all significant increase in the relative growth rate of the stems (Fig. 3*d*), but, o a relative basis, there is no significant difference for the position of the drop (Fig. 4*b*).

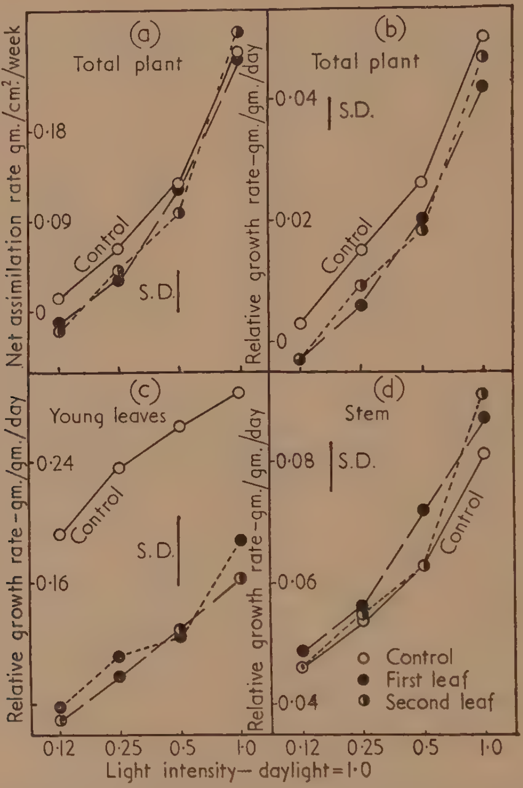

FIG. 3. Expt. 3. The influence of light intensity on the response of *H. annuus* to an application of 10 µg. of sodium 2:4-dichlorophenoxyacetate to either the first or second pair of leaves. The response measured in terms of (*a*) net assimilation rate, and the relative growth rates of (*b*) the whole plant, (*c*) the young leaves, and (*d*) the stem.

Once again the ratio of leaf area to leaf weight was significantly depressed by the phenoxyacetic acid treatment. The magnitude of the depression was of the same order at the three higher light intensities (*vide* Fig. 4*c*), but, at the lowest intensity, there was no significant effect.

II. *The effects of sodium 2:4-dichlorophenoxyacetate on the growth and development of plants receiving different light intensities prior to treatment*

In the previous experiments the plants were only subjected to a varyin light intensity after the application of the growth regulator. Thus the experiments provided no evidence as to whether the mode of physiological action would be modified if the plants had been grown under different light régimes before treatment with sodium 2:4-dichlorophenoxyacetate.

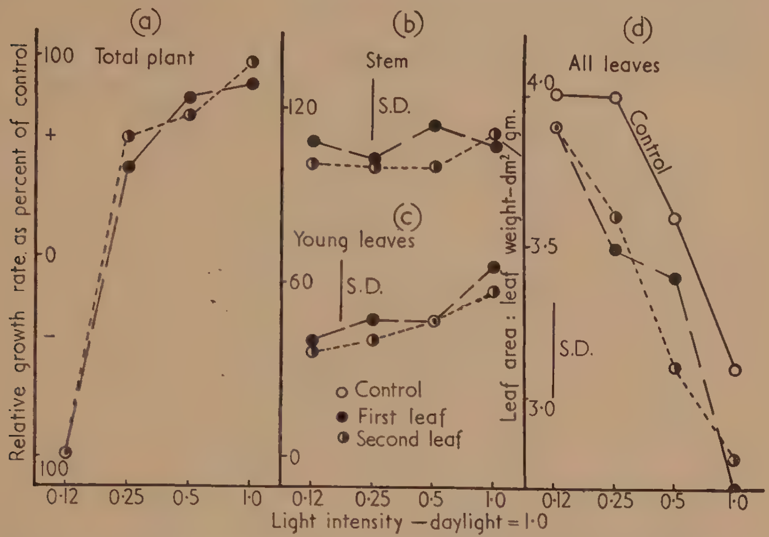

FIG. 4. Expt. 3. The influence of light intensity on the response of *H. annuus* to an application of 10 μg. of sodium 2:4-dichlorophenoxyacetate to either the first or second pair of leaves. Response expressed for (*a*) total plant, (*b*) stem, and (*c*) young leaves as percentage changes in the growth rate, relative to the controls. The effects on the ratio of leaf area to leaf weight are shown in (*d*).

To examine this aspect an experiment, similar in design to Expt. 2, was carried out, save that the plants were grown under the three levels of light intensity (1, 0·5, and 0·25 daylight) for 2·5 days prior to the application of the growth regulator. By this time the dry weights of the shaded plants were less than those of plants receiving full daylight and they already possessed some shade characteristics. However, at the time of application there was little difference in the lengths of the first leaves, each leaf being approximately 5 to 6 cm. long. Each treated plant received 50 μg. of the growth regulator applied as four droplets of 0·005 ml. of 0·25 per cent. aqueous solution. In this experiment the shoots only were harvested after 8 days, the stems being cut at the cotyledonary node. Separate determinations of dry weight were made for the first internode, the second internode, the first leaf pair, and the remaining leaves (young leaves), and from the resultant data the relative growth rates of each part and of the total shoot were calculated.

For all the criteria recorded there were highly significant differences between light intensities, but there was no significant interaction between light intensity and the effects of the phenoxyacetic acid. Application of the growth regulator had no significant over-all effect on the growth of the first leaf pair but caused a significant retardation of the total shoot ($P < 0{\cdot}01$), the first internode ($P < 0{\cdot}01$), and the young leaves ($P < 0{\cdot}001$)—Fig. 5. On the other hand, the relative growth rates of the second internodes were significantl ($P < 0{\cdot}001$) increased by the phenoxyacetic acid and relative to the contro changes the percentage increases were greater under shade. In no instance wa there a significant difference for droplet position, nor was there evidence o an interaction between light intensity and placement.

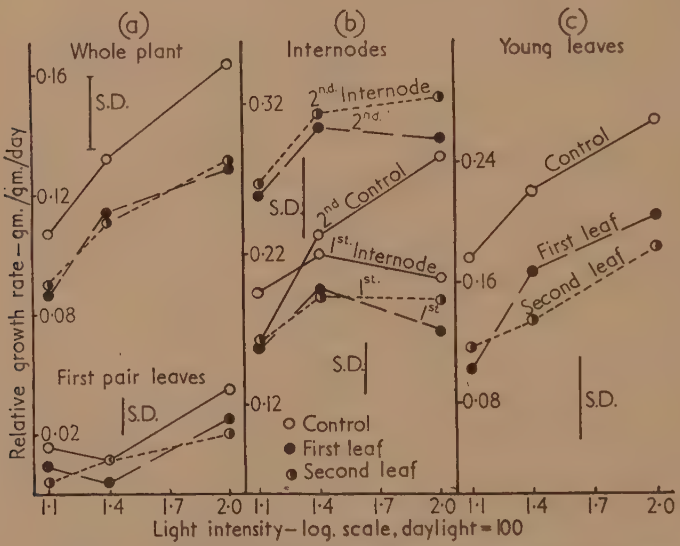

FIG. 5. Expt. 4. The influence of light intensity on the response of *H. annuus* to an application of 50 μg. of sodium 2:4-dichlorophenoxyacetate to either the first or second pair of leaves. Response measured in terms of changes in the relative growth rate of whole shoot and first pair of leaves (*a*), the first and second internodes (*b*), and young leaves (*c*).

III. *The effects of sodium 2:4-dichlorophenoxyacetate on the growth and development of plants transferred from one light level to another*

Since Expts. 3 and 4 were carried out at different seasons of the year, it seemed that more critical evidence of the relative importance of the light factor before or after the application of the growth regulator would be obtained if all the treatments were combined in a single experiment. For the first of these experiments half the pots were shaded (0·12 daylight) for 3 days prior to placing a droplet containing 5 μg. of the growth regulator on the midrib of each leaf of the first pair of leaves. Immediately afterwards the pots were transferred to 1·0, 0·25, or 0·12 daylight and harvested at the end of 7 days.

The addition of the growth regulator had no significant effect on either the relative growth rate of the whole plants or the net assimilation rate; see Fig. 6*b* and *a*. There was no evidence of an interaction between the effects of the growth regulator and the level of light intensity either prior to or after its application. At the same time there was no significant effect of the phenoxyacetic acid on the relative growth rates of the roots or hypocotyls.

The leaf-area ratio was significantly affected by the addition of the growth regulator and the interaction between compound and the light intensity prior to treatment was almost significant; see Fig. 6*c*. The trends for the ratio of leaf area to leaf weight were similar to those for the leaf-area ratio (Fig. 6*d*).

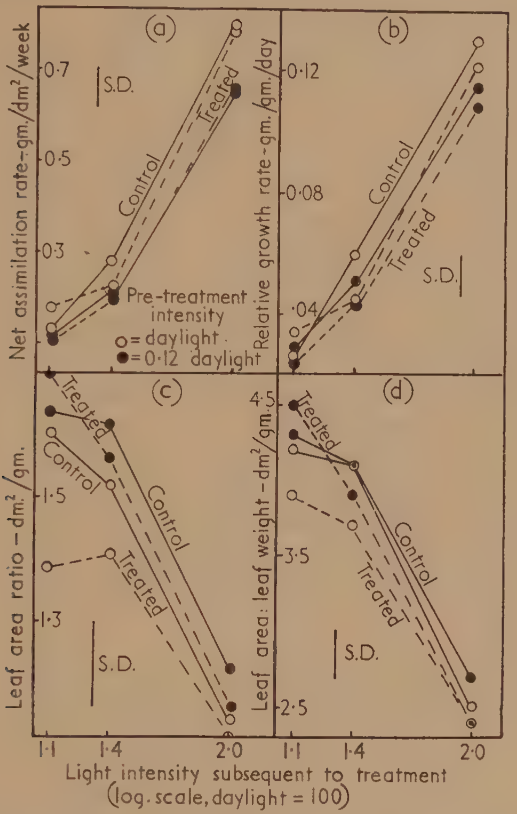

FIG. 6. Expt. 5. The effects of varying light intensity in the periods prior to and subsequent to treatment on the response of *H. annuus* to sodium 2:4-dichlorophenoxyacetate (10 μg. per plant). Response measured by changes in (*a*) net assimilation rate, (*b*) relative growth rate, (*c*) leaf-area ratio, and (*d*) ratio of leaf area to leaf weight.

The relative growth rate of the stem was enhanced by the phenoxyacetic acid, the greatest and just significant increase resulting from a combination of full daylight in the pretreatment phase together with 0·12 daylight after treatment (Fig. 7*a*). For the young leaves, either on a weight or area basis, the relative growth rates were greatly reduced by the phenoxyacetic acid. The order of the reduction was similar at all levels of light intensity in the post-treatment period, irrespective of the light level in the period prior to treatment (Fig. 7*b* and *c*). When these data are expressed on the usual percentage basis there is a significant effect of light intensity in the post-treatment period on area ($P < 0{\cdot}01$) and weight ($P = 0{\cdot}05$) due to the phenoxyacetic acid having a relatively greater effect as the intensity decreased.

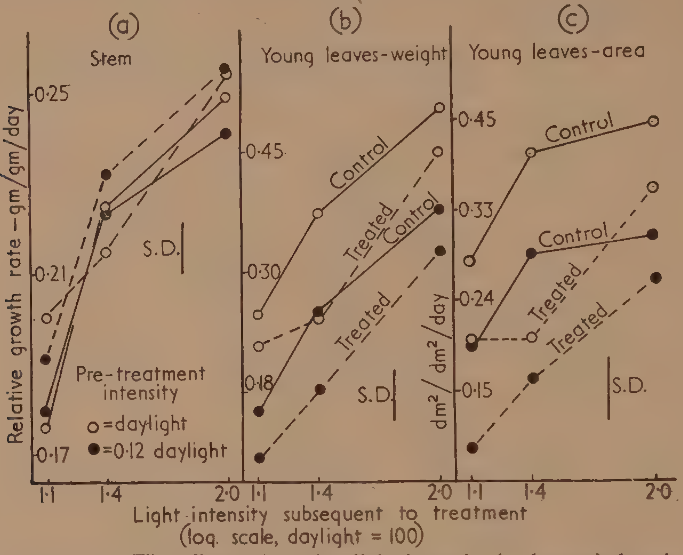

FIG. 7. Expt. 5. The effects of varying light intensity in the periods prior to and subsequent to treatment on the response of *H. annuus* to sodium 2:4-dichlorophenoxyacetate. Response expressed for (*a*) stem, (*b*) leaf weight, and (*c*) leaf area as percentage changes in the growth rates, relative to the controls.

TABLE V

Expt. 6. The effect of shading in the 3 days prior to the application of individual droplets of sodium 2:4-dichlorophenoxyacetate on the amount applied per gramme of shoot

	Dose (μg./g.) Light intensity prior to drop application	
Dose (μg./shoot)	1·0 daylight	0·12 daylight
12·5	164	231
25	328	463
50	656	926
100	1312	1851
200	2624	3704

In Expt. 5 the dose level was such that the growth rates of most of the parts, except for the young leaves, were not significantly affected by sodium 2:4-dichlorophenoxyacetate. It was decided therefore to repeat the experiment employing a range of 0, 12·5, 25, 50, 100, and 200 μg. per plant. In order to limit the size of the experiment to manageable proportions the same two levels of light intensity, namely 1·0 and 0·12 daylight, were employed in the periods prior to and after the droplets had been applied to the plant. Even with these limitations the experiment embraced 130 pots and it was not possible to recover the roots. Instead evaluation was in terms of the shoot above the cotyledons. The length of the pretreatment period was 3 days. The growth regulator was applied as a 0·25 per cent. solution of the sodium salt containing 0·28 per cent. of the surface active agent 'Tween 20'.

The mean dry weight of the initial samples which in the pretreatment period had received 1·0 and 0·12 daylight differed appreciably. Thus, when the dosages were calculated as the amount of growth regulator per unit dry weight of shoot, they were greater for the previously shaded plants; see Table V.

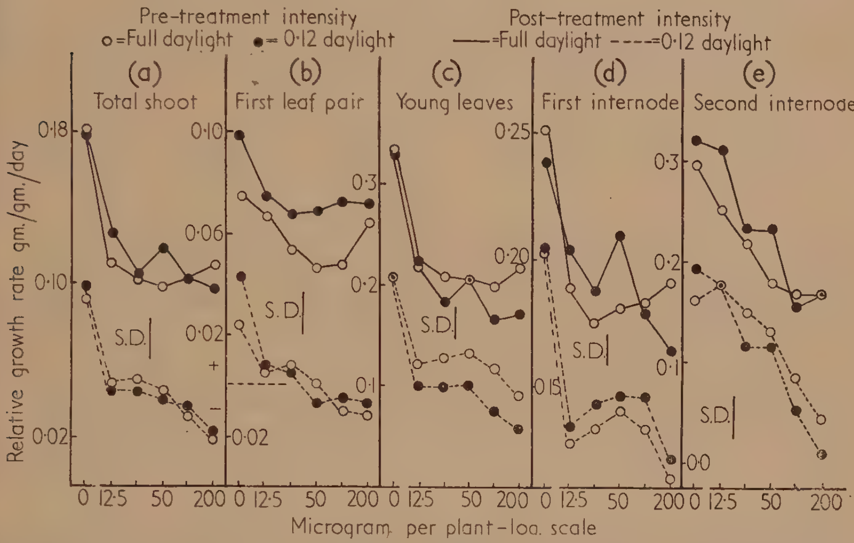

FIG. 8. Expt. 6. The effects of varying light intensity in the periods prior to and subsequent to treatment on the response of *H. annuus* to varying amounts of sodium 2:4-dichlorophenoxyacetate. Response measured by changes in the relative growth rates of (*a*) total shoot, (*b*) first pair of leaves, (*c*) second pair and younger leaves, (*d*) first internode, and (*e*) second internode.

Plants treated with 200 μg. were severely injured and towards the end of the 10-day period of the experiment some of the shaded plants had partially wilted and were very chlorotic, and would have died had they not been harvested. All plants treated with 50, 100, and 200 μg. developed local necrosis where the droplets were applied; the area of the necrotic centres increased slightly with both time and dose.

The dry weight of the shoots was compounded of separate measurements of the first pair of leaves, the young leaves, the first internode, and the second internode; the third and fourth internodes, being small, were included with the second. Many of the effects were highly significant, the general trends are shown in Fig. 8. Of the individual parts, except for the second internode, even 12·5 μg. caused a reduction of all the growth rates. With the second internode, however, the relative growth rate was progressively reduced by amounts greater than 25 μg.

In the post-treatment period all the relative growth rates for both the controls and the treated plants were greatly diminished at 0·12 daylight. In contrast, the differential effect of a variation in intensity in the pretreatment period was less consistent. At 0·12 daylight, in the period after application the rates of the first leaves were negative over the range 50–200 μg. per plant (Fig. 8*b*). These negative rates, together with the fact that for the corresponding controls the leaves grew very slowly, suggested that when the growth rates were expressed as percentages of the controls, the variance was not similar at the different levels of light intensity. Consequently, no analysis of variance of the percentage data was made for the first leaves. However, it is clear from Fig. 9*b* that the percentage reduction caused by all levels of the growth regulator was much greater for plants which had been shaded after the application.

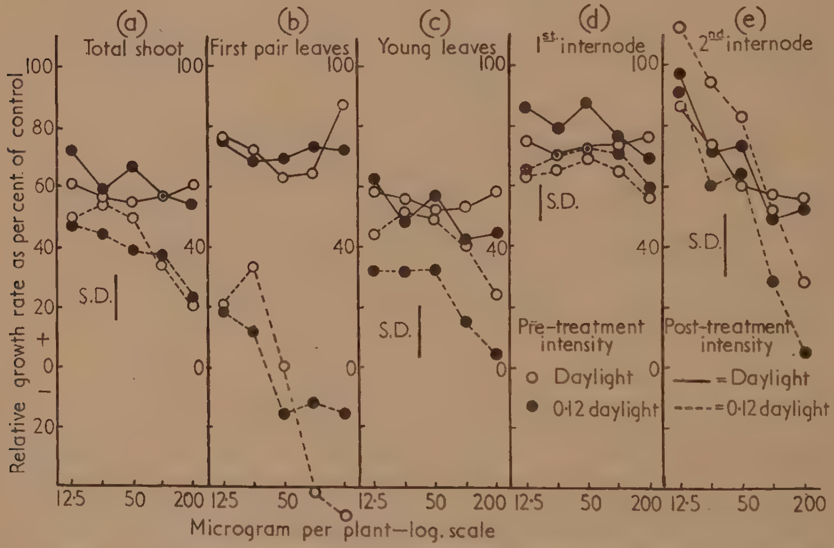

FIG. 9. Expt. 6. The effects of varying light intensity in the periods prior to and subsequent to treatment on the response of *H. annuus* to varying amounts of sodium 2:4-dichlorophenoxyacetate. Response expressed as changes in the growth rates, relative to the controls, of (*a*) total shoot, (*b*) first pair of leaves, (*c*) second pair and younger leaves, (*d*) first internode, and (*e*) second internode.

Relative to the control the percentage reductions in the total shoot caused by the phenoxyacetic acid were greater when the plants were shaded after treatment (Fig. 9*a*). In addition, there was a significant interaction ($P < 0{\cdot}01$) between the amount of the compound and the intensity in the post-treatment period; as the concentration increased, the progressive greater inhibition was greater in the shaded plants.

The percentage relative growth rates of the young leaves were also significantly depressed most by shading after treatment. There was also a significant interaction ($P < 0{\cdot}01$) between light intensities before and after treatment; i.e. when the plants were not shaded after the droplet application, then there was no differential effect of intensity prior to treatment, but when after the addition of the growth regulator the plants were shaded, shading in the first period led to greater depressions.

The percentage reductions of the first internode were less than those of the young leaves (Fig. 9*d*) except at 0·12 daylight prior to application, when they were greater. There was also a significant ($P < 0{\cdot}001$) difference between the effects of the intensities prior to treatment; in this instance, however, exposure to full daylight led to greater depressions.

Significant variation ($P < 0{\cdot}001$) of the second internode can be attributed to the pretreatment intensity, to dose, to the interaction of the light levels before and after treatment, and to the interaction of dose and intensity in the period after treatment (see Fig. 9*e*). This complex effect is such that when the dosage is small, the mean percentage inhibition of plants which are shaded after treatment is less than for plants receiving full daylight; the trend is reversed when the dosage is high. Moreover, in the post-treatment period shading combined with shading prior to the application induced the maximal inhibitory effects of the growth regulator.

It will be recalled that the plants which were initially shaded were smaller and in consequence in terms of the weight of the shoot they received relatively more material (see Table VI). Thus, some of the significant effects of varying light intensity before the droplet applications could be ascribed to variation in dose on a dry-matter basis. However, when the data for the young leaves and second internode were evaluated in this way the magnitude of the effects was reduced but not eliminated. Thus, the differential effects of the level of intensity in the first period cannot be accounted for solely by the difference in dosage.

In examining the interrelationships between the effects of the growth regulator and the level of light intensity before and after the application the recorded changes of parts distant from the point of droplet application must have involved factors of internal transport over considerable distances. It seemed therefore of interest to find out whether the general pattern was changed if an over-all application in the form of a spray was substituted.

Since it has been demonstrated that a reduction in light intensity causes considerable morphological changes in the shoot, it would be anticipated that such changes might alter the degree of retention of the spray droplets. Thus for valid comparisons possible differences in retention had to be estimated by the tartrazine method (see p. 180). From Table VI it is apparent that the amount retained per unit area was not greatly influenced by variations in the light level for 3 days prior to spraying. However, since the area per gramme was increased by shading, the amount retained per gramme of tissue was greater.

Eight days after the spray application the pots were harvested and the relative growth rates were calculated; the roots, however, were omitted since at 0·12 daylight the dry weights of the control plants showed no increase. For each part the relative growth rates were much higher for plants grown at the high level of light intensity. From Fig. 10 it is apparent that with increasing concentration there was a progressive depression in the growth of the whole plants and the first internode. There was a similar trend for the leaves, but there was no significant effect of the phenoxyacetic acid on the growth rate of the hypocotyl. In contrast for the second internode, save for the highest concentration, the growth was increased (Fig. 10*e*).

TABLE VI

Expt. 7. The effect of varying the light intensity prior to spraying, on the area and weight of H. annuus, and on the amount of spray retained

	1·0 daylight	0·12 daylight
Area (dm.^{2}/pot)	1·61	1·45
Weight (g./pot)	1·00	0·76
Volume retained (ml./pot)	1·02	0·94

	Amount retained (μg./g. of plant)	
Concentration of spray (p.p.m.)	1·0 daylight	0·12 daylight
12·5	12·8	15·5
25	25·5	31·0
50	51·0	62·0
100	102·0	124·0
200	204·0	247·0

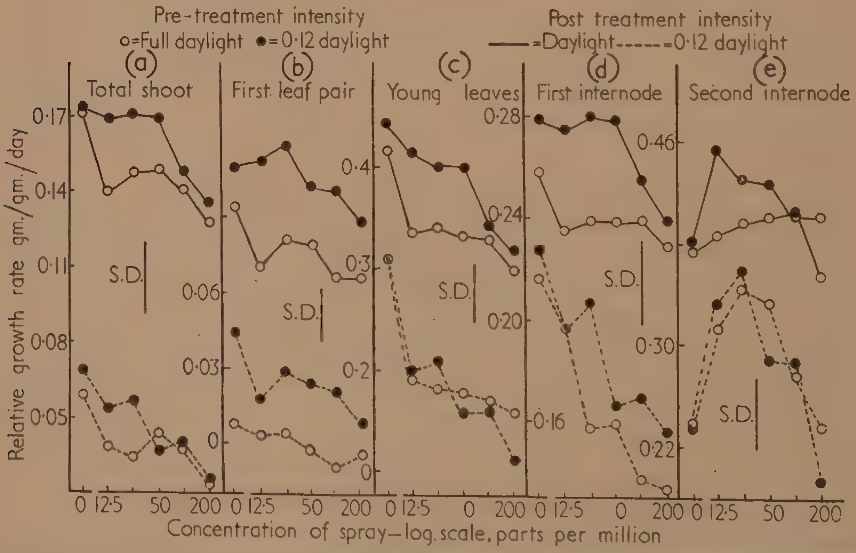

FIG. 10. Expt. 7. The effects of varying light intensity in the periods prior to and subsequent to treatment on the response of *H. annuus* to varying amounts of sodium 2:4-dichlorophenoxyacetate. Response measured by changes in the relative growth rates of (*a*) total shoot, (*b*) first pair of leaves, (*c*) second pair and younger leaves, (*d*) first internode, and (*e*) second internode.

When the level of light intensity after spraying was low, then the relative growth rate of the first leaves of control plants was small, while the rates were negative for plants sprayed at the higher concentrations. Thus, for the first leaves, when the growth rates were expressed as percentages of the controls, there was abnormally high variation and in view of this the calculations of the growth rates as percentages of the controls were based on the mean values (see Fig. 10*b*) and no statistical analysis was carried out.

When the results for the other parts of the plants are expressed relative to the controls, it is found firstly that for the young leaves the depression in growth decreases significantly ($P < 0{\cdot}001$) with increasing concentration and secondly that the percentage reductions are very much greater when after spraying the light intensity is 0·12 daylight (Fig. 11*c*). The development of the first internode is similarly affected, but there is, in addition, a significant effect of intensity in the pretreatment period (Fig. 11*d*).

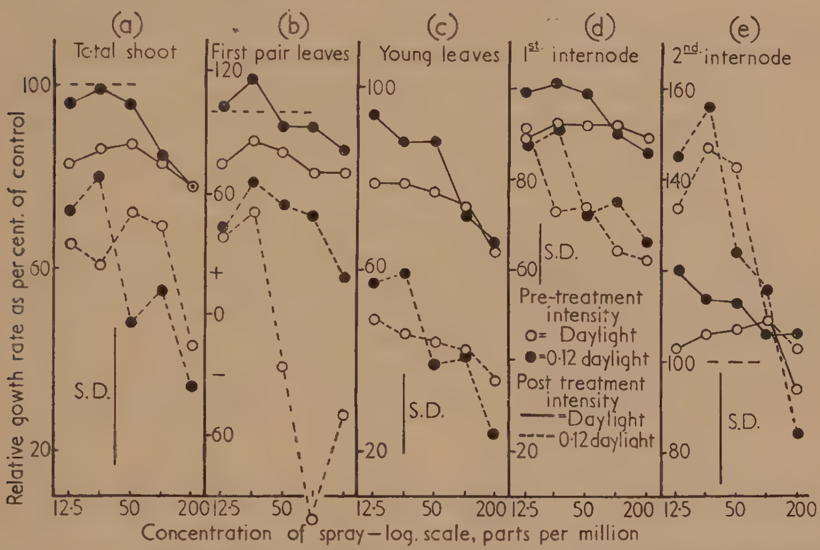

FIG. 11. Expt. 7. The effects of varying light intensity in the periods prior to and subsequent to treatment on the response of *H. annuus* to varying amounts of sodium 2:4-dichlorophenoxyacetate. Response expressed as changes in the growth rates, relative to the controls of (*a*) total shoot, (*b*) first pair of leaves, (*c*) second pair and younger leaves, (*d*) first internode, and (*e*) second internode.

Low concentrations of sodium 2:4-dichlorophenoxyacetate bring about large relative increases in the relative growth rate of the second internode (Fig. 11*e*). In this instance significant variation is due to both concentration and the light intensity after spraying ($P < 0{\cdot}001$), while the interaction is also significant ($P < 0{\cdot}01$). The interaction arises from the fact that, when the plants are shaded, there is a greater growth response at concentrations less than 100 p.p.m., whereas at 200 p.p.m. the relative growth of the internode is of the same order for plants receiving either 1·0 or 0·12 daylight.

In Expt. 6 it was noted that shaded plants when treated with the growth regulator showed such severe symptoms of toxicity that they were likely to die. It was decided, therefore, to investigate to what extents the effects of sodium 2:4-dichlorophenoxyacetate were dependent on the light intensity before and after spraying, when death is the criterion of response. The light intensities were similar to those in Expt. 7. Apart from the controls, these six concentrations of the spray solution (30, 60, 120, 240, 480, and 960 p.p.m.) were applied at a rate equivalent to 120 gallons per acre.

Signs of severe injury increased up to 4 weeks when many of the plants were dead, that is the base of the stem was discoloured and had lost all turgor. At the end of 5 weeks the experiment was terminated and final counts made of the number of dead plants per pot and individual probit lines fitted for each light intensity in the 6 blocks, i.e. 24 in all.

The residual heterogeneity was not significantly large, and there was no significant deviation from parallelism when all the probit lines were considered together. There were, however, significant differences in the positions of all four lines for the different combinations of light treatments. The degree of mortality was greatest when the plants were shaded before or after spraying. From Table VII it is clear that the effect of reducing the light intensity subsequent to rather than prior to spraying was greater.

TABLE VII

Expt. 8. The effect of varying the light intensity, before and after spraying on the concentration of sodium 2:4-dichlorophenoxyacetate required to bring about a 50 per cent. mortality (L.D. 50)

Light intensity (fractions of daylight)			
Pretreatment	Post-treatment	L.D. 50 (p.p.m.)	Lower and upper fiducial limits ($P = 0{\cdot}05$) of L.D. 50 (p.p.m.)
1·0	1·0	157	135–84
1·0	0·12	26	21–32
0·12	1·0	95	81–101
0·12	0·12	16	10–21

IV. *The effect of light intensity on the absorption and translocation of 2:4-dichloro-5-iodophenoxyacetic acid*

In the complementary paper on *L. minor* (Blackman and Robertson-Cuninghame, 1955) previous work on the factors limiting the rate of penetration of 2:4-dichlorophenoxyacetic acid and its subsequent transport within the plant have already been reviewed. It has been shown that the translocation of growth regulators was associated with the movement of carbohydrates from the leaves and, as a result, translocation was dependent upon the level of light intensity. However, these findings were not strictly applicable to the conditions in the present investigation. Moreover, it was clear that any satisfactory evaluation of the effects of light intensity on the response of *H. annuus* necessitated a knowledge of the effect of altering the light intensity on both penetration and translocation of 2:4-dichlorophenoxyacetic acid.

It was apparent that the most sensitive means of determining penetration and translocation would have been to employ a sample of 2:4-dichlorophenoxyacetic acid incorporating radioactive carbon, preferably within the benzene ring. At the time of this investigation this requirement could not be met and it was decided to use 2:4-dichloro-5-iodophenoxyacetic acid containing radioactive iodine (I^{131}). Admittedly this compound was not strictly comparable, but nevertheless other experiments had shown that the two compounds produced similar effects on *H. annuus*.

The short half-life (8 days) of I^{131} made it necessary to plan short-term experiments and to commence all experiments as soon as possible after receiving the compound. A limited amount of 0·1 per cent. solution with a low activity (0·0025 mC./ml.) was available some time before the main sample and this was used for preliminary tests.

Droplets of the radioactive material were applied to the first leaf in the normal way and 40 hours later the leaf surface was washed three times with water and the washings collected while the treated leaf and the rest of the plant were macerated and extracted by the method already described. The first washing and the first extraction accounted for 88 per cent. of the material applied, while by the time the leaf had been rewashed and the plant tissue had been extracted three times the residual tissue showed negligible radioactivity. Thus this experiment indicated that there was no strong adsorption of any iodo compound. To confirm this point a large amount of the active material was added to an alcoholic solution in which macerated tissue from untreated plants was suspended. Some hours later the pad of tissue formed by centrifuging after washing with alcohol was found to contain no I^{131}. On the basis of these apparently satisfactory techniques the main experiment was started.

The design was of the normal type for investigating the relationships of the effects of varying light intensity before and after the application of the droplets. During the 2-day period prior to the application two levels of light intensity were employed, viz. the normal intensity of the greenhouse (high level) and 12 per cent. of this intensity (low level), and the same two levels were used in the post-treatment period. The application consisted of 0·025 ml. of a 0·1 per cent. solution containing, in addition, 0·5 per cent. of 'Tween 20', i.e. approximately 800 $m\mu C$.

Pots from each group were harvested on five occasions after treatment (3, 6, 12, 24, and 48 hours). At the time of harvesting the treated leaf was removed from each plant and the labelled compound that had not been absorbed was washed off with two separate lots of water. Shortly after harvesting, the remaining portions of the plants were separated so that they could be extracted independently.

The amounts of active material absorbed by the leaf are seen in Fig. 12*a*. An analysis of variance showed that highly significant variation was due to the sampling occasion and to the level of light intensity after the application, while the interaction between time and intensity was also significant. There was no significant effect of the light level prior to the application.

This experiment indicated that when a hygroscopic surface active agent is added to the aqueous solution penetration is not complete at the end of 48 hours. A second similar experiment was therefore carried out in which the sampling period was greatly extended and a comparison made of the variation in penetration with and without the addition of the same surface active agent to the aqueous solution. The experiment was conducted without shading in the greenhouse, and the results are shown in Fig. 12*b*. It is seen that after 2 days very little further penetration takes place from an aqueous solution. In contrast, penetration is still continuing after 7 days when the solution is both hygroscopic and of a low surface tension.

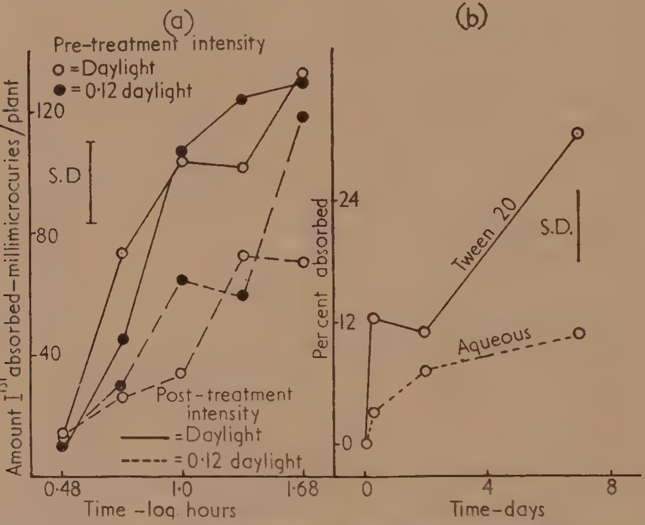

FIG. 12. Expt. 9. (*a*) The influence of light intensity in the period prior to and subsequent to treatment on the absorption of sodium 2:4:5-dichloroiodophenoxyacetate containing radioactive I^{131} by the leaves of *H. annuus*. Expt. 10. (*b*) The effect of including a surface active agent (Tween 20) in an aqueous solution on the amount absorbed.

In order to investigate the movement of the material that had been absorbed a balance-sheet was drawn up of I^{131} found in the washings and the various extracts for each part. Somewhat surprisingly, in view of the preliminary trials, it was found that a considerable amount of the active material could not be accounted for, and since the loss was not discovered until the experiment had been completed, it was not possible to investigate how much of an iodo compound remained in the macerated tissues. However, some extracted material of both leaf and stem from this and another experiment was available and was found to contain relatively high activity which did not equilibrate with alcohol, nor could it be extracted with acetone, with acidified alcohol, 5N hydrochloric acid, or 2N alkali.

This unexpected failure to account for all the active material reduces the value of the results, for it is not certain whether each component of the plant adsorbed the same proportion of the active material. Thus it must be concluded for this compound that, using an alcoholic extraction procedure, a liquid assay technique is not suitable for estimating the amount of I^{131} in plant tissue.

However, since active material was recovered it is established that I^{131} was transported to various organs. Three hours after application a negligible amount had appeared in tissue other than that of the treated leaf, while after 6 hours a small amount had reached the parts below the treated leaf; see Table VIII.

TABLE VIII

Expt. 9. Balance sheet showing distribution of I^{131} *in H. annuus after sodium 2:4-dichloro-5-iodo*^{131}*-phenoxyacetate had been applied to one of the first pair of leaves of plants receiving a high and low light intensity either before or after the application*

Light intensity		Time	I^{131} (mμC) (Total for 8 plants) (Amount applied: 6,400 mμC)										
Before	After	(hours)	Total absorbed	Treated leaf	Roots	Hypocotyl	First internode	Second internode	Second leaf	Third leaf	Opposite leaf	Total translocated	Loss
High	High	6	558	356	52			0			0	52	150
Low	High	,,	356	202	6			2			0	8	146
High	Low	,,	211	150	13			0			0	13	48
Low	Low	,,	238	138	4			0			0	4	90
High	High	12	823	573	45			12			0	57	193
Low	High	,,	847	586	47			17			0	64	197
High	Low	,,	268	280	46			1			0	47	−59
Low	Low	,,	508	268	13			7			0	20	220
High	High	24	804	542	2	10	60	18	48		0	138	124
Low	High	,,	984	545	1	23	42	10	64		0	140	299
High	Low	,,	576	338	0	26	20	3	23		0	72	94
High	High	48	1,054	398	1	18	66	35	147	89	3	359	297
Low	High	,,	1,035	360	1	32	100	19	187	73	2	414	261
High	Low	,,	563	321	0	2	39	14	41	27	0	123	119
Low	Low	,,	943	444	0	6	104	18	55	12	1	196	303

(Accuracy of estimation is in the order of 5%. The roots, hypocotyl, and first internode are below the treated leaf, while the second internode and the second and third leaves are above it.)

At the end of 12 hours active material was detected above the treated leaf as well as below it. The amount recovered in the various parts of the shoot increased with time, but at the end of 24 hours there was no I^{131} in the opposite leaf and, in the plants which were shaded after the application, none in the roots, while only a very small amount was recorded in the roots of the corresponding unshaded plants.

Greater quantities of I^{131} were observed in all these organs after 48 hours and there was a small amount in the opposite leaves of plants receiving a high intensity after the application.

On the basis of the results for the quantities absorbed and the amounts present in the treated leaf the general trends indicate that in the period of post-treatment transport from the treated leaf to the shoot is greater at a high intensity. On the other hand, variation in the light level prior to the application appears to have much less effect.

In Expt. 9 after 48 hours little I^{131} had appeared in the roots or the leaf opposite the treated leaf. Expt. 10, for which the penetration data have already been described (e.g. Fig. 12*b*), confirms that even at the end of 7 days the roots and the opposite leaves may have little or no activity; see Table IX. Irrespective of the amounts absorbed there is considerable accumulation in the hypocotyl and the second and third pair of leaves, but the relative distribution between the first and second internodes is dependent on the quantity penetrating the treated leaf.

TABLE IX

Expt. 10. The effects of the addition of a hygroscopic surface active agent to the solution on the penetration of sodium 2:4-dichloro-5-iodo^{131}*-phenoxyacetate and the subsequent distribution of* I^{131} *in the plant*

	Amounts of I^{131} recovered as % of that applied					
	Aqueous solution			Solution plus surface active agent		
	Time (hours)					
	6	24	168	6	24	168
Total absorbed	3·10	7·21	10·94	12·51	11·10	30·47
Treated leaf	1·12	1·72	6·21	8·48	4·59	10·59
Root	0	0	0	0	0	0·04
Hypocotyl	0	0·12	0·96	0·24	0·39	1·45
1st internode	0·04	0·08	0·04	0·27	0·55	3·49
2nd internode	0	0·12	0·16	0·08	0·31	1·06
Opposite leaf	0	0	0·08	0	0	0·08
2nd and 3rd leaves	0	0·36	0·92	0·08	1·88	2·39
Total translocation	0·04	0·68	2·16	0·67	3·13	8·51

It could be postulated that the radiation from the I^{131} caused damage to the tissues such that the growth regulator would act differently from a non-isotopic compound. A subsidiary experiment was therefore carried out in which plants were treated with 25 μg of sodium 2:4-dichloro-5-iodo-phenoxyacetate with and without radioactive iodine, and their development was compared with that of both untreated plants and others treated with 5 μg of sodium 2:4-dichlorophenoxyacetate. The conditions of application were the same as in Expt. 9. From Table X it is clear that the relative growth rates of the treated leaves and the young leaves were depressed by all the compounds but there was no evidence suggesting a dissimilarity between the effects of the two iodo compounds.

TABLE X

The effect of sodium 2:4-dichlorophenoxyacetate, sodium 2:4-dichloro-5-iodo-phenoxyacetate, and sodium 2:4-dichloro-5-iodo^{131}*-phenoxyacetate on the relative growth rate of the young leaves and the treated leaf of H. annuus*

Treatment	Dose (μg./plant)	Relative growth rate (g./g./day) Young leaves	Relative growth rate (g./g./day) Treated leaf
Control	0	0·263	0·074
Sodium 2:4-dichlorophenoxyacetate	5	0·203	0·058
Sodium 2:4-dichloro-5-iodo-phenoxyacetate	25	0·239	0·065
Sodium 2:4-dichloro-5-iodo^{131}-phenoxyacetate	25	0·232	0·066
Sig. diff. ($P = 0·05$)	—	0·032	0·008

DISCUSSION

In the investigation relating to *Lemna minor* (Blackman and Robertson-Cuninghame, 1955) it was found that irrespective of the light intensity a concentration of sodium 2:4-dichlorophenoxyacetate which produced small but significant decreases in the relative growth rates did not cause comparable reductions in the net assimilation rate. A similar trend is shown for *H. annuus* under conditions where the amount of the growth regulator only resulted in small depressions in the relative growth rate (Expts. 1, 2, 3). Even when the assimilation rate tended to be depressed it is debatable whether this decrease can be attributed to direct interference with the photosynthetic processes but rather can be ascribed to the abnormal leaf movements. Within a few hours of the application the leaves assumed a downwards vertical position and only over a considerable period resumed a horizontal position and normal phototropic responses.

On the basis of these results, as in the case of *L. minor*, it can be concluded that the primary action of this growth regulator in initiating depressions in growth cannot operate through a direct action on the mechanisms of carbon fixation. Under conditions where the dosages reach a level eventually causing death or major depressions in the growth rate which are associated with a yellowing of the leaves and an abnormal leaf structure (Watson, 1948) it would be expected that the photosynthetic rate would be affected. In this connexion Loustalot and Muzik (1953) working with *Stilozolobium deeringianum* and Wedding, Erickson, and Brannaman (1954) using either infiltrated leaf disks of orange leaves or sterile cultures of *Chlorella pyranoidosa* have recorded marked depressions in photosynthesis.

It can be shown mathematically that the relative growth rate of the whole plant is the product of the net assimilation rate and the leaf-area ratio. Thus, if the growth rate is depressed but the net assimilation rate is relatively unaffected by a given amount of the dichlorophenoxyacetic acid, it follows that the leaf-area ratio must be decreased. This interrelationship is illustrated in Fig. 1 for Expt. 1 and comparable data for Expts. 2 and 3 are given in Table XI. From the results of these three experiments it is therefore apparent that irrespective of the light level the depression in the growth rate induced by the growth regulator is largely dependent on reductions in the leaf-area ratio.

TABLE XI

Expts. 2 and 3. Interrelationships between changes in the leaf-area ratio brought about by combinations of varying light intensity and the application of sodium 2:4-dichlorophenoxyacetate to either the first or second leaves

		Final leaf-area ratio—cm.^{2}/g. Light intensity—daylight = 1·0			
		1·0	0·5	0·25	0·12
Expt. 2.	Control	150	182	213	—
	1st leaf	114	149	174	—
	2nd leaf	114	148	175	—
Expt. 3.	Control	160	186	211	194
	1st leaf	123	153	163	173
	2nd leaf	128	146	151	171

The depressive effects on the leaf-area ratio can be analysed more precisely by considering the effects on the component parts of the plant. From Tables II and IV it is evident that at each intensity the phenoxyacetic acid decreases the proportion by weight of leaves, increases the proportion of stem, but has either little effect on the ratio of roots (Table II) or tends to increase the ratio in daylight (Table IV). It is also apparent that the ratio of leaf area to leaf weight is generally reduced at all light levels (e.g. Figs. 1*d*, 2*c*, 4*c*), and it therefore follows that the reduction in leaf area due to the growth regulator is compounded of a decrease in leaf weight and a smaller area per unit weight of leaf.

One of the most striking features of the results of Expts. 1–4 is that for both control and treated plants the characteristic plastic responses to falling light intensity, such as the increases in proportion of stem, the leaf-area ratio, and the ratio of leaf weight to leaf area, follow very similar trends. It will, however, be recalled that though at each light intensity the individual plants received the same amount of growth regulator, on the basis of the results of Expt. 9 (Fig. 12*a*) the amount penetrating into leaves will have been somewhat less under the conditions of shade. Therefore it could be advanced that the proportionate changes in leaf and stem compared to those in full daylight were being produced by a smaller amount of compound.

That there are marked interactions between 0·12 and full daylight and the changes induced by somewhat larger amounts of the growth regulator are clearly brought out in Expts. 6–8. Whether the criterion is death (Table VII) or the depression in shoot growth (Figs. 8 and 10), the effects of a given amount of compound are relatively much greater if the plants receive 0·12 daylight after the application. In Expt. 8 there is the additional residual effect of the intensity prior to the application, namely, that for previously shaded plants a smaller concentration of the spray solution is required to bring about a 50 per cent. mortality. However, taking into account the results of Expt. 7 (Table VI), the greater susceptibility may at least in part be attributed to an enhanced retention of the spray solution due to the larger leaf-area ratio. In Expt. 6 there is some suggestion, even when allowance is made for the reduced size of the shaded plants (Table V), that the largest depressions in shoot growth induced by 12·5–50 μg. per plant arise when the plants are shaded both before and after the application.

On the basis of existing knowledge it would seem that any physiological interpretation of the present results must remain largely speculative. Some of the difficulties which arise in seeking to explain the adaptive changes of the sunflower following on an alteration in light level have already been discussed (Blackman and Wilson, 1954), while the complexity of assessing the interacting effects of indolylacetic acid and 2:4-dichlorophenoxyacetic acid on the growth of *H. annuus* has been stressed (Blackman and Robertson-Cuninghame, 1954). Since these papers were written reviews which in part relate to the auxin control of phototropic responses (Brauner, 1954) and the role of light in the formation or inactivation of auxins (Gordon, 1954) serve to emphasize the complexities.

Largely on the basis of *in vitro* studies it has been established that indolylacetic acid can be destroyed by extracts from etiolated pea stems, bean roots, and the apex from green pineapple plants, e.g. Galston, Bonner, and Baker (1953), Wagenknecht and Burris (1950), and Gortner and Kent (1953). It is still debatable whether the same oxidase system exists in the three species since the optimum pH for the reaction varies, and whereas Mn^{++} ions have proved to be inhibitory in pea extracts they are accelerating at low concentrations in bean and pineapple extracts. It would seem that oxidation takes place via a peroxidase-hydrogen peroxide system with which is associated an inhibitor deactivated by blue light, a possible photoreceptor being a flavo-protein. On the other hand, Tang and Bonner (1948) have demonstrated that indolylacetic acid is destroyed if the extracts are made from pea seedlings grown in the dark but not if the plants are raised in the light owing to the presence of an inhibitor. Moreover, these authors, by infiltrating indolylacetic acid into etiolated pea stem segments, obtained evidence of direct oxidation. Thus, it is apparent that on the basis of these findings it is difficult to assess how far an alteration in light level affects the balance between the levels of inhibitor and the oxidase system in whole plants.

When etiolated pea seedlings are illuminated within bands of a 100 Å, Parker, Hendricks, Borthwick, and Went (1949) found that on an equal energy basis a wavelength of 6,500 Å was at least 10 times as effective as 5,800 Å or 4,350 Å in accelerating leaf expansion as measured in terms of length. At 5,800 Å as the intensity was increased the leaves became progressively longer but the length of the second internode was slowly reduced. If pea seedlings are first grown in the dark and then illuminated with red light and segments from the epicotyl placed in a range of concentrations of indolylacetic acid, the maximum extension growth produced is of the same order as that found for segments from plants grown in complete darkness, but on the other hand the concentration of auxin required to produce maximal extension is some 10 to 100 times as great (Galston and Baker, 1953). The effect of red light vanishes if segments are cut more than 24 hours after the exposure and is maximal if the interval is 18 hours. Using *Avena* coleoptiles Liverman and Bonner (1953) record that irrespective of whether the sections are floated on solutions during or after a brief exposure to red light, the subsequent extension growth in the dark, in contrast to that of pea segments, is greater than that of sections maintained in the dark. If sections are first treated with red light and then exposed to infra-red light, the extension-promoting properties of red light are reversed, but this counteracting effect only takes place if at the time of exposure to the infra-red light the segments have already been placed in the solutions of indolylacetic acid. When exposure to red light is omitted, infra-red light is without effect.

Although much attention in the last few years has been focused on the extension growth of stem tissues, few comparable papers have appeared on leaf expansion. Went (1951) in his review postulated that the factors responsible for the growth of the mesophyll differ from those relating to the growth of the veins and petiole. Indolylacetic acid can increase vein growth without affecting mesophyll expansion, which is positively related to increasing light intensity. The experiments of Miller (1951) support the suggestion that the factors for leaf expansion may be different. When disks, punched out of etiolated bean leaves, are floated on a solution containing glucose and potassium nitrate, exposion to white light (200 foot-candles for 15 minutes) increases leaf expansion. Similar expansions can be obtained by substituting for the light treatment metalic ions in the culture solutions, of which Co^{++} produces the maximum effect while Mn^{++} is inactive.

Against the background of this new knowledge it is evident that in any final evaluation of the interactions between light intensity and the effects of 2:4-dichlorophenoxyacetic acid consideration must be given to at least two light-sensitive systems concerned with auxin balance in the shoot and a set of factors, as yet ill defined, relating to leaf growth and expansion. There is the further difficulty that in many investigations of light effects comparisons have been made between plants grown in darkness and those given intensities, which, relative to normal daylight, are very low. For example, while Parker *et al.* (1949) record that leaf expansion of peas is increased as the intensity increases, it has been found by one of us (G. E. B.) that reducing the intensity from daylight to 0·5 daylight increases the leaf area of the pea and that reductions in area only take place at lower intensities. In other words, the relationship is curvilinear.

In the simplest analysis changes in the growth rates of leaves or stem induced by varying light intensity may be looked on as changes in the balance between the levels of auxins concerned with cell division and cell extension and the competitive power of the component parts for essential substrates for the full differentiation of the tissues. With this changing pattern of growth and the associated differences in cell physiology it is not unexpected that the influence of 2:4-dichlorophenoxyacetic acid should show interactions with light intensity.

Robinson and Brown (1952), by taking serial sections from the tip of a bean root, have demonstrated that between the meristematic phase and that of completed cell extension there are ordered changes in the protein content and the enzyme activities of dipeptidase, glycine-oxidase, phosphatase, and invertase. More recently Galston and Dalberg (1954) have examined the distribution of indolylacetic acid oxidase in etiolated pea shoots and found that *in vitro* the level of oxidase was lowest in the extracts from parts showing rapid extension growth and highest in tissues which had ceased to extend. When tissue from zones which had either stopped extending or were extending rapidly were placed in a solution containing indolylacetic acid, it was found that some loss of the auxin from the solution could be detected almost immediately from the older tissue but that there was for the younger tissue a lag period of 10–20 minutes. Galston and Dalberg have interpreted this lag as being the time taken for the adaptive formation of the oxidase on the grounds that the disappearance from the external solution can be used to calculate *in vivo* activity of the oxidase. They further postulated that in *green*, not etiolated, intact plants (see p. 203) the auxin control of growth could be exercised through the balance between the level of indolylacetic acid and the adaptively formed oxidase. Their initial hypothesis carries the implication, for which no proof is provided, that the differences between tissues in the initial rate of uptake are wholly and solely dependent on differences in the rate of *in vivo* oxidation, since (i) the possibilities of physical absorption or other metabolic pathways or (ii) oxidation, either at the cut surfaces or diffusion of the oxidase into the external solution, are not discussed. On the basis of existing knowledge it seems most improbable that for such large pieces (3 mm. long) an adaptive enzyme could be formed in so brief an interval: even in bacteria such short times have rarely been recorded. Moreover, Reinhold (1954) has investigated by time-course studies the uptake of indolylacetic acid by disks of carrots and segments of the third internode of etiolated pea shoots (var. 'Alaska') under conditions where oxidation at the cut surfaces or through the secretion of oxidases were of little account. It was observed that an initial phase of sharply declining rate of uptake was followed by a period when the rate of uptake was relatively constant. As a result of studying the effects of a wide range of enzyme inhibitors it was concluded that the uptake is in part related to physical absorption and in part metabolic; the first phase represents the attainment of physical equilibrium, the second is determined by a metabolic process.

Goldacre, Galston, and Weintraub (1953) observed that in brei from etiolated pea epicotyls the oxidation of indolylacetic acid is enhanced in the presence of 2:4-dichlorophenol and that the enhancement is less when the preparations are made from plants receiving red light. If, therefore, 2:4-dichlorophenoxyacetic acid is broken down into the parent phenol and the rate of breakdown is linked with the level of light received, the greater depressive effects of the growth regulator on the leaves and first internode at 0·12 daylight could be ascribed to a greater depression in the level of indolylacetic acid. On the other hand, the results for peas may not apply to *H. annuus* since Gortner and Kent (1953) found that in pineapple extracts the dichlorophenol was without influence on the oxidation of indolylacetic acid. Moreover, if the release of the phenol from the phenoxyacetic acid is a primary factor in producing physiological effects, it would be expected that variations in the activity of substituted phenoxyacetic acids would be directly linked to variations in the toxicity of the corresponding phenols, and for *L. minor* this does not appear to be so (Blackman and Robertson-Cuninghame, 1954).

The results of Expts. 6 and 7 emphasize that the interactions between the effects induced by the growth regulator and the level of light intensity are greatest when there is a wide divergence in the intensity during the period after the application, namely 1·0 and 0·12 daylight. From Figs. 9 and 11 it is apparent that the largest percentage reductions of the first and second pairs of leaves and of the first internode occur under conditions of shade and that compared to the decreases induced in plants receiving full daylight the differences are maximal for the first leaf pair and least for the first internode. In contrast, the effects on the second internode are quite different. In Expt. 6 for the smaller amounts of the growth regulator the reactions are little affected by a change in light intensity, while in Expt. 7 when the plants are shaded the growth rates relative to that of the control are increased—not decreased.

If it is postulated that the sole function of 2:4-dichlorophenoxyacetic acid is to suppress the available supply of endogenous auxins, then when the supply is limiting growth increasing amounts of the exogenous growth regulator should progressively depress growth, but when the level of auxins is supra-optimal and inhibitory, additions of the exogenous compound could at first increase growth. If, on the other hand, it is advanced that the endogenous and exogenous growth substances have additive effects, then provided that the supply of natural auxins is limiting, the net result would at first be an increase in growth followed by retardation, as the level of the exogenous compound rises. Alternatively, when the supply is optimal or supra-optimal, then any addition of 2:4-dichlorophenoxyacetic acid would be suppressive. The first postulate could explain the recorded changes for both pairs of leaves and the first internode if it is further assumed that while in full daylight the level of endogenous auxins is *circa* optimal it is greatly reduced by shading. The second hypothesis would demand that shading raises the level from optimal to supra-optimal and inhibitory. To make either hypothesis fit the results for the second internode demands that a reduction in the light intensity has contrasting effects on the auxin level of this internode. On an additive basis it would have to be advanced that shading reduces the supply of endogenous auxins to a sub-optimal concentration, while if the exogenous growth regulator is wholly antagonistic, this would imply that a reduction in intensity shifted the level from optimal to supra-optimal.

Before either of these hypotheses can be considered tenable it is necessary to fit within the framework the changes brought about by the differences in light level in the period prior to the application of the growth regulator. Taking first the first internode and the postulate of antagonism between endogenous and exogenous compounds, it must be assumed that shading before treatment in contrast to shading after treatment increases the natural auxin level since whether the plants are shaded or not after the application the order of growth depression is less for the initially shaded plants (Figs. 9 and 11). Similarly, on the basis of additive effects it must be argued that shading prior to treatment shifts the auxin concentration from supra-optimal towards optimal.

The same difficulties of postulating reverse effects of shading before and after the application also arise from the reactions of both types of leaves in Expt. 7 (Fig. 10); while in Expt. 6, since there are no significant effects of the intensity prior to treatment, the supposition is demanded that the endogenous auxin supply is affected by shading after the application but not before.

The position is even more complex for the growth of the second internode in Expt. 7 (Fig. 11), since the needs of either hypothesis require that when the plants are shaded after treatment shading before treatment has no influence on the availability of the endogenous auxins, but that under a combination of shading followed by full daylight the supply is increased on the basis of antagonism or reduced on the concept of additive effects.

From the foregoing considerations it is clear that the observed interactions between light intensity and the effects induced by the dichlorophenoxyacetic acid cannot be satisfactorily interpreted on the simple assumptions that the changes in light level bring about comparable changes in the availability of the endogenous auxins in the several parts and that the primary action of the growth regulator is either to inactivate or supplement the natural auxin supply. That in the sunflower more than one mechanism is involved has already been concluded from previous studies of the interaction between indolylacetic acid and 2:4-dichlorophenoxyacetic acid (Blackman and Robertson-Cuninghame, 1954), and in this discussion it has already been suggested that the pattern of the mode of action may change with differences in the physiological status of the cells, particularly in relation to differentiating and differentiated cells.

The order of the relative growth rates reflects to some extent the proportion of new tissue that is being formed, and in all experiments it has been found that the relative growth rates of the leaves and internodes are markedly depressed by intensities below 0·5 daylight. Thus, it could be advanced that the enhanced depressive effects of the phenoxyacetic acid under conditions of shade are associated with reductions in the growth rate and the consequent changes in the ratio of differentiating and differentiated cells. In both Expts. 6 and 7 it has been seen that when the plants are shaded after the application the percentage depressions induced in the first pair of leaves are much greater than those for the younger leaves, and inspection of Figs. 8 and 10 shows that for the control plants the growth rate of the older leaves is far less. In other words, the maximal reductions occur when the rate of leaf growth is minimal. Considering next the residual effects of the initial light intensity from Fig. 10, it is apparent that where shading prior to treatment has subsequently increased the growth rates of either class of leaf, then the percentage depressions due to growth regulator are less (Fig. 11). When the results for the first internode are examined in the same way, the decreases in the growth rate due to shading after treatment are matched by larger percentage reductions induced by the growth regulator. In Expt. 7 the significant influence of the light intensity prior to treatment cannot be satisfactorily interpreted, since while a change in intensity exerts little influence on the growth rate, it affects the level of depression. There are similar disparities for the second internode. In Expt. 7, if the plants are shaded after treatment the reduction in the growth rate is associated with an acceleration in growth due to the dichlorophenoxyacetic acid, but when the plants receive full daylight in the second period the comparable acceleration due to the initial shading takes place without any change in the growth rate of the control plants.

In seeking to interpret the interaction between the effects of the growth regulator and light intensity, there was a seeming contradiction (p. 207) in that either on the basis of an antagonistic or additive mode of action it was necessary to postulate that shading before or after the application had reverse effects on the availability of endogenous auxins in the leaves. It has, however, been pointed out in the previous paragraph that shading in the first period tends to increase the growth rate and in the second to depress it. It could therefore be concluded that in the leaves the effects of 2:4-dichlorophenoxyacetic acid are dependent on the rate of growth with which is associated a changing pattern of endogenous auxins. Moreover, since the exogenous auxin always depresses growth and since it is reasonable to suppose that the rate of growth is positively related to auxin level, it can also be concluded that the action of the phenoxyacetic acid is antagonistic rather than additive.

For the first and second internodes, the interrelationships between changes in the light intensity and changes in growth caused by the dichlorophenoxyacetic acid cannot be linked satisfactorily to comparable changes in the growth rates, more particularly in relation to the residual influences of intensity in the initial period. On the other hand, inspection of Figs. 5, 7, and 9 shows that in full daylight the growth rate of the second internode is appreciably greater than that of the first internode, and it could therefore be advanced that the divergences in the reaction to the growth regulator are related to differences in physiological status due to the differing rate of growth. However, under conditions of shade in Expts. 6 and 7 for the control plants the growth rates of both internodes are similar, while in Expt. 4 the growth rate of the first internode is less at 0·5 daylight and more at 0·25 daylight, and yet it has been seen that the reactions of the two internodes to the growth regulator are markedly divergent. This lack of agreement might be taken as invalidating any relationship between the growth rate and the physiological action of the growth regulator, but it is stressed that the rate of growth is a very inadequate measure of physiological differences between the internodes. On general grounds it is likely that in the second internode there is a higher proportion of meristematic and vacuolating cells, and in consequence an equivalent rate of growth of the first internode will be based on a smaller production of new cells offset by larger increases in weight due to the differentiation of cells which have ceased to expand. Similarly decreases in the growth rate due to shading are unlikely to bring about *pro rata* decreases in new cell formation since fully extended cells will be less differentiated.

From the experimental results it is clear that as the light intensity is reduced the net assimilation rate is markedly depressed, and in consequence the rate of growth of the whole plant is limited by the supply of carbon substrates demanded for cell production and differentiation. It might therefore be concluded that this limitation rather than the changing pattern of endogenous auxins is a more important factor. It may well be that the interaction between the dichlorophenoxyacetic acid and light intensity may be linked directly with the redistribution of available substrates to the component parts. From Fig. 11 it is evident that at 0·12 daylight the redistribution of substrates which leads to a greater proportionate growth of the stem can be affected by additions of the dichlorophenoxyacetic acid, for in the second internode the growth rate has been increased by as much as 56 per cent. Whether this increased growth results from a direct effect of the growth regulator by interfering with the supply of the available substrates or whether the balance is changed by a differential increase in new cell formation is debatable. It is, however, suggested that the second supposition is more important.

There is one other factor which may contribute to the observed divergences between the internodes, namely, a marked differential accumulation of 2:4-dichlorophenoxyacetic acid. Although the results of the transport experiments are not precise, the data of Table VIII indicate that in shaded plants the content of I^{131} is somewhat higher in the first internode. Thus, postulating in the first instance that the two tissues have a comparable auxin status, for equivalent amounts of the phenoxyacetic acid the effects on the first internode should be accentuated. In Expt. 6 this is not so since the mean reductions in the growth rate of the second internode are much greater. Again, in Expt. 7, where there was an overall spray application with less likelihood of localized accumulation, 15·5 μg. caused some reduction in the first internode but 247 μg. were required to bring about a reduction in the second internode, and there is no indication from Table VIII that the differential accumulation is 16 times as great in the first internode.

On balance the data of Tables VIII and IX suggest that in full daylight the first internode contains more I^{131} and in Expts. 5 and 7 the gains in growth of the second internode as against the small depressions in the first could be attributed to a variation in accumulation. On the other hand, in Expt. 6 with increasing amounts of the growth regulator the order of the depression is accentuated more in the second internode.

In conclusion, while the present investigation has established with some precision the relationships between light intensity and the effects of 2:4-dichlorophenoxyacetic acid on the growth of the whole plant and its component parts, the basic principles underlying the nature of the interrelationships still remain obscure. This obscurity clearly rests on the complexity of the factors involved, coupled with the varying reactions of the first and younger pairs of leaves and the different internodes. It has been put forward that the observed effects on leaf growth can in part be explained on the twin assumptions that the growth regulator suppresses the availability of endogenous auxins and that the mode of action is linked with the physiological status of the tissues as indicated by the growth rate, but these two postulates fail to account for the reaction of the internodes even when differential accumulation is taken into account. An explanation could be provided by the introduction of more variables, but the question still remains of what variables. There is evidence that the dichlorophenoxyacetic acid may have both antagonistic and additive modes of action, but it would still be necessary to postulate that these actions are related to the level of the light intensity. It might be that the system is affected by a change in the level of indolylacetic acid due to the influence of blue light on the balance of oxidases and inhibitors. It might be that the effectiveness of indolylacetic acid is influenced by a change in the energy levels of red and infra-red light. It might equally well be advanced that indolylacetic acid *per se* may not be the principal factor since in full daylight previous experiments have suggested that the growth of the leaves and internodes of the sunflower are all depressed by additions of this auxin (Blackman and Robertson-Cuninghame, 1954). There are still, too, the problems of what are the factors which control the differentiation of fully extended cells. Certainly, the present experiments have shown that 2:4-dichlorophenoxyacetic acid can only modify and not dominate the plastic responses, and the associated changes in structure, which occur with a change in light intensity. Until a greater integration has taken place between experiments on isolated tissues and those on whole plants it would be idle to speculate further.

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The Oxidation of d-*iso*Citrate by Pea-seedling Mitochondria

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SUMMARY

A diphosphopyridine nucleotide specific *iso*citric dehydrogenase has been isolated from pea-seedling mitochondria and purified 10-fold. In the presence of manganese the enzyme catalyses the oxidative decarboxylation of d-*iso*citrate to α-oxoglutarate and CO_2, but under similar conditions does not catalyse the reverse reaction. Evidence is presented indicating that –SH groups of the enzyme are essential for activity. The properties of the enzyme are described.

INTRODUCTION

THE work of Adler, Euler, Günther, and Plass (1939) established the triphosphopyridine nucleotide (TPN) specificity of *iso*citric dehydrogenases in animal tissues and this specificity has been confirmed for *iso*citric dehydrogenase of plant origin (Berger and Avery, 1944; Ceithmal and Vennesland, 1949; Whatley, 1951). Kornberg and Pricer (1951) have isolated two *iso*citric dehydrogenases from yeast, one being specific for TPN, the other for diphosphopyridine nucleotide (DPN), and raised the possibility of the general occurrence of the DPN specific enzyme. Davies (1952, 1954) has shown that DPN stimulates the oxidation of *iso*citrate by mitochondria isolated from pea seedlings and suggested that mitochondria contain a DPN specific dehydrogenase. This paper deals with the partial purification and properties of the enzyme.

Since this work was completed Plaut and Sung (1954) have reported the purification of a DPN specific *iso*citric dehydrogenase from heart mitochondria.

METHODS

Chemicals. d-*Iso*citrate isolated from blackberries was a gift from Mr. D. Williamson; α-oxoglutarate was presented by Mr. R. Hems. dl-*iso*Citrate was prepared according to Pucher and Vickery (1946); DPN (50 per cent. pure) according to Le Page (Umbreit, Burris, and Stauffer, 1949); DPN (95 per cent. pure) and TPN (60–95 per cent. pure) according to Kornberg and Pricer (1953) and Kornberg and Horecker (1953); aluminium hydroxide Cγ according to Bertho and Grassmann (1933); crystalline alcohol dehydrogenase from 'Premier' yeast according to Racker (1950*a*); glucose-6-phosphate dehydrogenase according to Kornberg (1950).

Analytical. Spectrophotometric measurements were made at laboratory temperature with a Unicam SP. 500 spectrophotometer. Reduced DPN was determined by measurement of the absorption at 340 mμ, using the extinction coefficient $6{\cdot}22 \times 10^6$ cm.^{2} mole^{-1} (Horecker and Kornberg, 1948). α-oxo-Glutarate was isolated as its 2: 4-dinitrophenylhydrazone by filter-paper chromatography (Seligson and Shapiro, 1952) and estimated by the method of Friedemann and Haugen (1943). Protein was determined by the method of Warburg and Christian (1942).

Enzyme assays. DPN *iso*citric dehydrogenase was assayed at a wavelength of 340 mμ in glass or silica cells of light path 1 cm., containing DPN (0·001 M.), $MnCl_2$ (0·0015 M.), d-*iso*citrate (0·005 M.), cysteine (0·005 M.), tris (tris(hydroxymethyl)aminomethane) pH 7·4 (0·025 M.), and enzyme in a total volume of 3 ml. Under these conditions 1 unit is defined as the amount of enzyme producing an optical density change of 0·01 per minute. For the assay of TPN *iso*citric dehydrogenase, TPN replaced DPN in the above system. Fumarase and aconitase were assayed by the method of Racker (1950*b*).

RESULTS

1. *Preparation of an extract of pea mitochondria.* Mitochondria isolated from pea epicotyls (100 g.) by the method of Millerd, Bonner, Axelrod, and Bandurski (1951) were suspended in 15 ml. of 0·1 M.$NaHCO_3$ and disintegrated by shaking for 5 minutes with Chance Ballotini Glass beads (size 14) in a Mickle shaker. The suspension was centrifuged at 24,500×g. for 30 minutes in an International refrigerated centrifuge and the supernatant collected to give 12 ml. of a clear extract. All operations were carried out at 0° C.

2. *Demonstration of DPN* iso*citric dehydrogenase.* The extract contained an enzyme which reduced DPN in the presence of *iso*citrate (Fig. 1). However, after standing at 0° C. the preparation showed a lag period before reduction of DPN occurred, the length of this period depending on the period of storage of the extract (Fig. 2). Though no certain explanation of the lag can be given, the lag can be eliminated by the addition of reducing agents such as cysteine, ascorbic acid, glutathione, and potassium cyanide and may be induced in a fresh extract by the addition of dilute hydrogen peroxide (Fig. 3). These results suggest that the enzyme is inactive in the oxidized state and requires –SH groups for activity.

The products of the reaction between DPN and *iso*citrate were shown to be α-oxoglutarate and H_2DPN, by isolating the 2: 4-dinitrophenylhydrazone of α-oxoglutarate on a filter-paper chromatogram by the method of Seligson and Shapiro (1952) and by oxidizing the H_2DPN with acetaldehyde in the presence of DPN specific alcohol dehydrogenase (Fig. 4). Analysis established

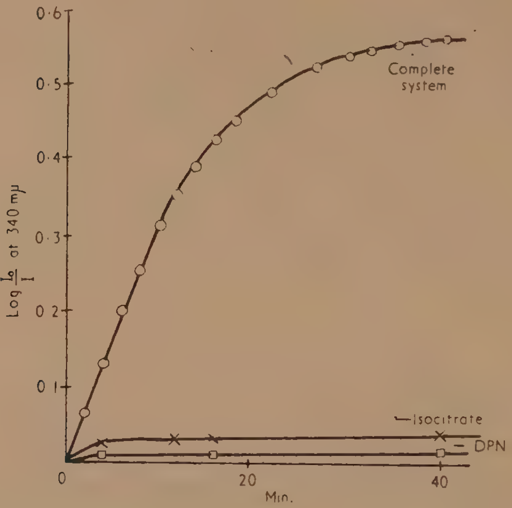

FIG. 1. Spectrophotometric demonstration of the reduction of DPN by *iso*citrate in the presence of an extract of pea mitochondria. Cells contained enzyme, *iso*citrate (0·005 M.), DPN (250 μg.), tris buffer pH 7·4 (0·025 M.), and $MnCl_2$ (0·0015 M.) in a total volume of 3 ml., with omissions as indicated.

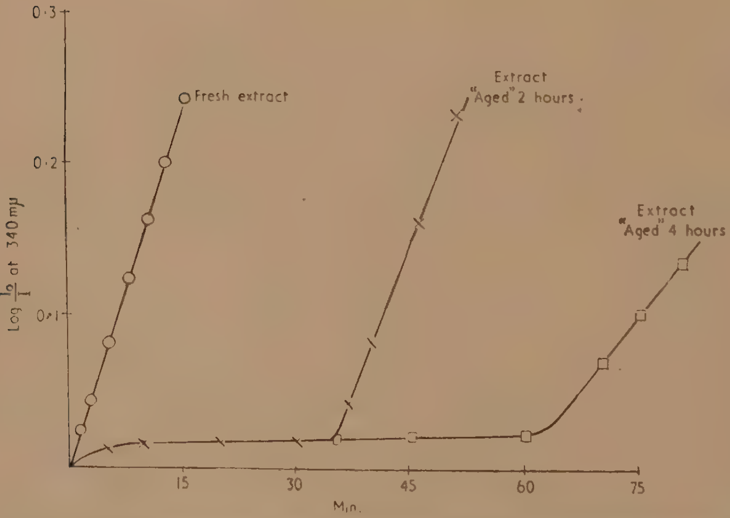

FIG. 2. Effect of storage at 0° on the activity of DPN *iso*citric dehydrogenase present in an extract of pea mitochondria. Contents of cells as in Fig. 1.

the stoichiometry of the reaction (Table I) and permits formulation of the reaction observed in the equation

$$\text{Isocitrate} + \text{DPN} \rightleftharpoons \alpha\text{-Oxoglutarate} + CO_2 + H_2\text{DPN}.$$

The extract also possessed TPN *iso*citric dehydrogenase activity, and

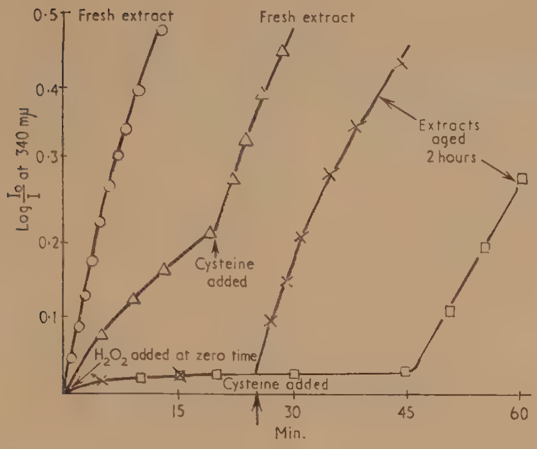

FIG. 3. Effect of cysteine and hydrogen peroxide on the DPN *iso*citric dehydrogenase activity of fresh and 'aged' extracts of pea mitochondria. Contents of the cells as in assay system with the omission of cysteine. Hydrogen peroxide added *c.* 5 μmoles, cysteine added 30 μmoles.

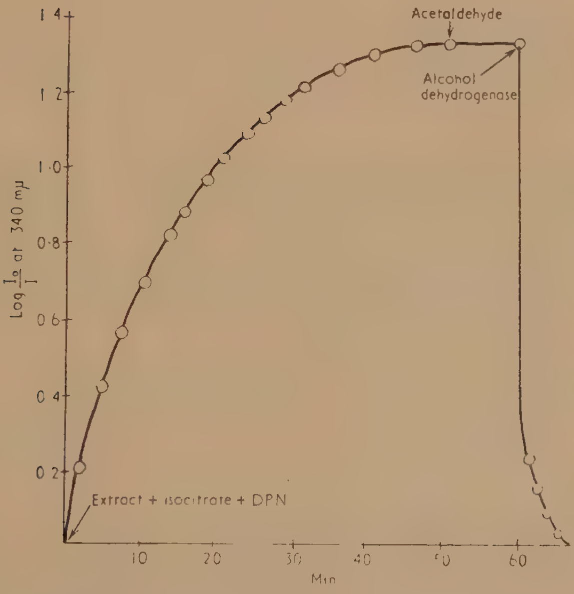

FIG. 4. Spectrophotometric demonstration of H_2DPN formed by the reduction of DPN by *iso*citrate in the presence of an extract of pea mitochondria. Contents of the cell as in assay system except that the DPN added was 500 μg. After 50 minutes acetaldehyde (0·3 ml. containing 4 μmoles) was added, followed after 10 minutes by alcohol dehydrogenase.

TABLE I

Stoichiometry of the reaction between d-isocitrate and DPN

Reaction carried out in 1-cm. cells containing assay system. After reading optical density at 340 mμ reaction stopped by tipping into 1 ml. 30 per cent. trichloroacetic acid. Analysis as in text. Data refer to 3-ml. solution.

Enzyme preparation	DPN used (μmoles)	α-oxoglutarate formed (μmoles)
Extract	0·91	0·85
Ammonium sulphate precipitate .	0·79	0·80
Cγ eluate	0·48	0·46

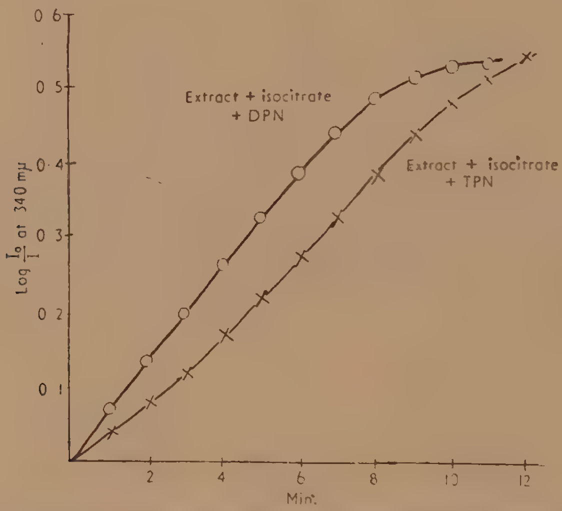

FIG. 5. Reduction of DPN and TPN by *iso*citrate in the presence of an extract of pea mitochondria. Cell contents as in Fig. 1 with the addition of cysteine (0·005 M.) and the replacement of DPN by TPN in cell *B*.

though TPN activity was less than the DPN activity (Fig. 5), there remained the possibility that the observed reaction with DPN was due to the presence of a pyridine-nucleotide transhydrogenase (Kaplan, Colowick, and Neufeld, 1953).

$$\textit{Iso}\text{citrate} + \text{TPN} \longleftrightarrow \alpha\text{-Oxoglutarate} + \text{CO}_2 + \text{H}_2\text{TPN},$$

$$\text{H}_2\text{TPN} + \text{DPN} \longleftrightarrow \text{TPN} + \text{H}_2\text{DPN},$$

$$\text{Sum: } \textit{Iso}\text{citrate} + \text{DPN} \longrightarrow \alpha\text{-Oxoglutarate} + \text{CO}_2 + \text{H}_2\text{DPN}.$$

To eliminate this possibility the enzyme was purified.

3. *Purification of DPN* iso*citric dehydrogenase.* Ammonium sulphate (2·5 g.) was dissolved in 12 ml. of the extract and the precipitate collected by centrifuging for 5 minutes at 24,500×g. The precipitate was dissolved in 5 ml. water and alumina Cγ (2 ml. containing 20 mg./ml. dry wt.) added. After standing for 3 minutes the alumina was collected by centrifugation for 2 minutes at 10,000×g. and then suspended in 5 ml. 0·1 M. phosphate buffer

pH 7·4. After standing for 2 minutes the suspension was centrifuged for 2 minutes at 10,000 × g. to give a clear supernatant containing the enzyme. A second elution of the alumina gave a solution containing the enzyme with a somewhat lower specific activity. All operations were carried out at 0° C. The purification achieved by this procedure is shown in Table II.

TABLE II

Purification of DPN iso*citric dehydrogenase*

Starting material 100 g. pea epicotyl. DPN and TPN *iso*citric dehydrogenase assayed as in text. Specific activity is the number of units per mg. protein. A unit of fumarase or aconitase is amount of enzyme producing an optical density change of 0·001 per minute under conditions specified by Racker (1950*b*).

	DPN *Iso*citric dehydrogenase			TPN *Iso*citric dehydrogenase		Fumarase	Aconitase
Step	Total units	Specific activity	Per cent. recovery	Total units	Specific activity	Total units	Total units
Extract . . .	1,432	7·0	100	994	4·8	8,640	2,680
Ammonium sulphate precipitate	497	8·3	34	5	0·8	0	0
1st eluate from Cγ .	245	68	17	0	0	0	0
2nd eluate from Cγ .	159	45	11	0	0	0	0

4. *Properties of the enzyme.* The Cγ eluate contained the enzyme in the oxidized state and it was necessary to add a reducing agent to activate the enzyme. Cysteine was the most effective reducing agent used (Table III) and the effect of cysteine concentration is shown in Table IV. The enzyme

TABLE III

Effect of reducing agents on the activity of DPN iso*citric dehydrogenase*

Activity measured 15 seconds after addition of enzyme to assay system, except cysteine replaced by reducing agent indicated in table. All reducing agents added to give final concentration 2×10^{-3} M.

	Reducing agents added				
	None	Cysteine	Glutathione	Ascorbic acid	KCN
Activity ($\Delta \log I_0/I$ in 2 min. at 340 mμ) .	0	0·15	0·05	0·11	0·6

TABLE IV

Effect of cysteine concentration on the activity of DPN iso*citric dehydrogenase*

Activity measured 15 seconds after addition of enzyme to assay system, except concentration of cysteine varied as in table.

Concentration of cysteine (moles per litre)	$8{\cdot}3 \times 10^{-3}$	$4{\cdot}2 \times 10^{-3}$	$10{\cdot}4 \times 10^{-3}$	$2{\cdot}2 \times 10^{-4}$	0
Activity ($\Delta \log I_0/I$ in 2 min. at 340 mμ) .	0·09	0·088	0·072	0·06	0

was very unstable and the substrates, DPN and *iso*citrate supplied separately, provided only slight protection (Table V). Because of the instability of the

TABLE V

Stability of DPN iso*citric dehydrogenase at 0° C.*

Activity measured 15 seconds after addition of enzyme solution, containing substances in table, to assay system.

	Additions				
	None	Cysteine (2×10^{-3} M.)	d-*iso*Citrate (10^{-3} M.)	DPN (10^{-3} M.)	Serum albumin 5 mg./ml.
Per cent. loss of activity after 90 min. at 0° .	35	40	27	25	32

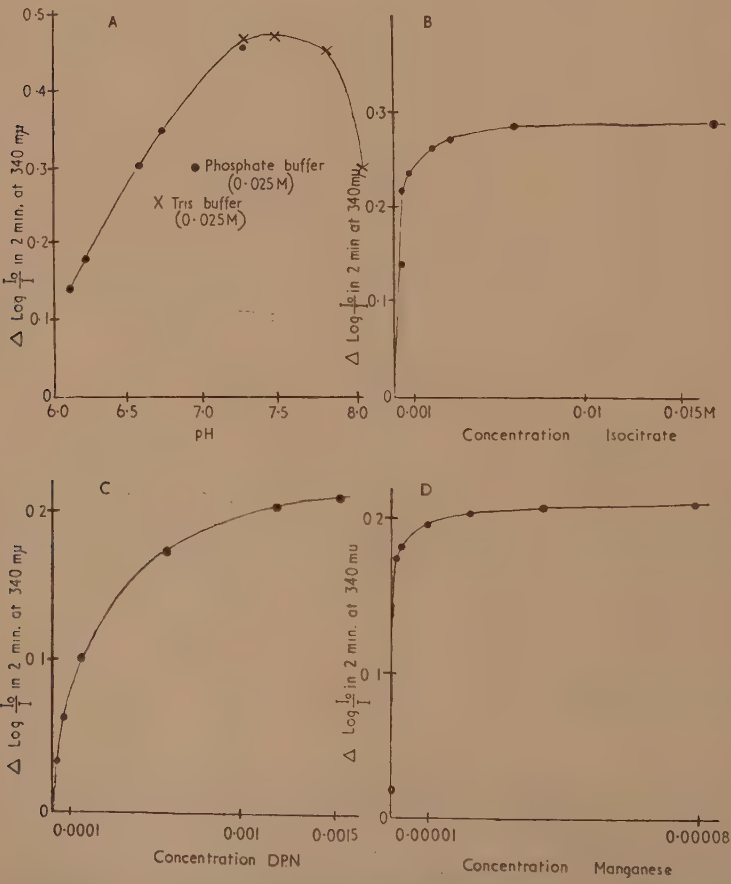

FIG. 6. Properties of purified DPN *iso*citrate dehydrogenase. A. Effect of pH on the activity of the enzyme. B. Effect of *iso*citrate concentration. C. Effect of DPN concentration. D. Effect of manganese concentration. Cell contents as in the assay system with one component varied through the range indicated in the figures.

enzyme, it was essential when studying the properties of the enzyme to work rapidly. By means of the spectrophotometric method it was possible to measure the initial rates of reaction in the first 2–3 minutes and by using all positions in the cell carrier of the spectrophotometer to complete the measurement of 9 initial rates in 10–12 minutes.

The pH activity curve of the enzyme is shown in Fig. 6A. The effect of *iso*citrate concentration is shown in Fig. 6B. The Michaelis constant is *c.* 3×10^{-4}, which is considerably higher than the Michaelis constant of TPN *iso*citric dehydrogenase. The Michaelis constant for DPN calculated from the data of Fig. 6C is *c.* $1{\cdot}5 \times 10^{-4}$. Fig. 6D illustrates the effect of manganese concentration. The purified preparation will not oxidize H_2DPN in the presence of α-oxoglutarate and CO_2, though the initial extract and the ammonium sulphate fraction appear to do so (Fig. 7). However, these fractions contain glutamic dehydrogenase and the observed disappearance of H_2DPN may be due to the formation of glutamic acid from α-oxoglutarate and ammonia, present in the preparation.

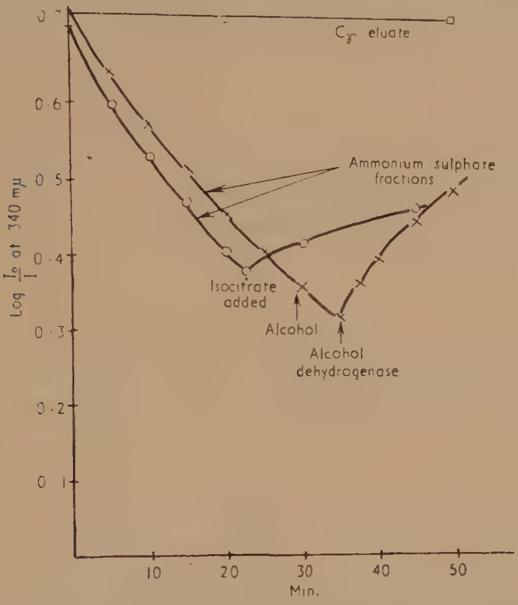

FIG. 7. Oxidation of H_2DPN by α-oxoglutarate in the presence of DPN *iso*citric dehydrogenase purified by ammonium sulphate fractionation and failure of the Cγ eluate to effect the same reaction. Silica cells contained enzyme, α-oxoglutarate (0·05 M.), $NaHCO_3$ buffer pH 7·4, H_2DPN (250 μg.), cysteine (0·005 M.), and $MnCl_2$ (0·0015 M.) in a total volume of 3 ml.

DISCUSSION

The existence of two enzymes in higher plants involved in the oxidation of malate is well established (Vennesland and Conn, 1952). The presence of two enzymes involved in the oxidation of *iso*citrate raises the question of the biological significance of these enzymes. With the establishment of a DPN specific *iso*citric dehydrogenase it is apparent that all the pyridine-nucleotide enzymes involved in the tricarboxylic acid cycle can function with DPN. TPN *iso*citric dehydrogenase and the malic enzyme of Ochoa have been implicated in carbon dioxide fixation (Ochoa, 1951). Since CO_2 fixation in malate and *iso*citrate is essentially the reversal of two steps in the tricarboxylic acid cycle, it is possible that the existence of DPN and TPN specific enzymes permits the oxidation of substrates by the tricarboxylic acid cycle and the simultaneous fixation of CO_2 in substrates of the cycle by reductive carboxylation.

The failure to observe the reductive carboxylation of α-oxoglutarate confirms the findings of Kornberg and Pricer (1951) and Plaut and Sung (1954), and supports the view of Kornberg and Pricer (1951) that oxalosuccinate may not be an intermediate in the oxidation of *iso*citrate by the DPN specific enzyme.

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Glucose Respiration in *Zygorhyncus moelleri*; the Entry of Glucose into the Cells

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SUMMARY

When glucose was added to carbohydrate-starved cells of *Zygorhyncus moelleri* the rate of oxygen uptake did not immediately rise to a constant value, but there was a lag period of 2 or 3 hours before it reached its maximum level. The length of this lag period increased from a few minutes for short periods of starvation to 2–3 hours after 12 hours in a carbohydrate-free medium. Factors believed to affect cellular permeability (a cationic detergent, adjustments of the pH, and of the potassium/calcium ratio) reduced the length of the lag period by not more than 40 per cent. of the original value without affecting the final rate of oxygen uptake. Investigation of the entry of glucose into the cells showed that the rate of oxygen uptake was not limited by the concentration of intra-cellular glucose for more that about 11 per cent. of the lag period in starved cells. The reasons for this difference in the percentage of the lag period apparently due to a permeability barrier are tentatively discussed in connexion with the route by which glucose enters the cells.

When glucose was added to cells of *Zygorhyncus moelleri* grown on glucose and ammonium chloride, and then starved for 24 hours in a carbohydrate-free medium, there was a lag period of 2 to 3 hours before the rate of oxygen uptake became constant. Investigation of this phenomenon showed that in the cells starved of carbohydrate, but not of nitrogen, glucose oxidation was stimulated by the presence of nitrogen-containing substances remaining in the cells (Moses, 1954). This stimulation could be abolished by azide. However, even when azide was present, there still remained a considerable lag period, and further work was undertaken to examine the possibility that a low permeability of the cell membrane to glucose might also be a contributory factor.

METHODS

Preparation of cell suspensions for measurement of respiration. The technique used for the growth and starvation of *Z. moelleri* and the preparation of the material for investigation of the respiration in the Warburg microrespirometer apparatus have been described in an earlier paper (Moses, 1954). Briefly, this consisted of inoculating 22 ml. of a glucose-ammonium chloride medium with 10 ml. of a heavy spore suspension obtained from a culture of the organism on agar. The spore suspension was filtered through glass wool before inoculation to remove gross pieces of mycelium. The inoculated medium was shaken

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for 18 hours in a Warburg water-bath at 25°. The cells were then centrifuged, washed, resuspended in 25 ml. of phosphate buffer, pH 6·8, and shaken for 24 hours in the water-bath to starve them. At the end of this period the cells were centrifuged and washed, and normally suspended in 35 ml. of 0·1 M. phosphate buffer, pH 6·8. Samples (2 ml.) of this suspension (containing 1·5–2·0 mg. dry weight of cells per ml.) were dispensed into each Warburg flask. The cells used in this work were nitrogen-starved. In such cells no stimulation due to nitrogenous substance takes place when glucose is added unless an external source of nitrogen is also added (Moses, 1954).

When the effect of pH was investigated, the starved cells, after washing, were suspended in 17·5 ml. of distilled water, 1 ml. being dispensed into each Warburg flask together with 1 ml. of 0·2 M. phosphate buffer of the required pH. In experiments investigating the effect of altering the potassium/calcium ratio in the medium the cells were suspended in water and no buffer was used. A preliminary experiment showed that the final rate of oxygen uptake with glucose as substrate was the same whether the cells were suspended in water or phosphate buffer (pH 6·8), and that the pH of an aqueous suspension did not fall during the course of glucose metabolism. Varying potassium/calcium ratios were obtained by mixing different quantities of potassium chloride and calcium chloride; the final concentration of the salt mixture was 0·066 M. Unless otherwise stated, 0·3 ml. of a 4 per cent. (w/v) glucose solution (i.e. 66·7 μmoles of glucose) was added to the cells from the side-arms of the Warburg flasks. Carbon dioxide was absorbed by 0·2 ml. of 15 per cent. (w/v) potassium hydroxide in the centre wells, and the total liquid volume in the Warburg flasks was made up with water to 3·0 ml. (excluding the alkali).

Glucose determinations. Glucose was determined as reducing sugar by the method of Hagedorn and Jensen (1923), as modified by Hanes (1929) and Hulme and Narain (1931). As some of the glucose samples were present in ethanol solution, an ethanol blank of appropriate concentration was used in these analyses. Paper chromatographic analysis of the specimens reserved for glucose estimations showed that glucose was the only reducing substance present as detected by an ammoniacal silver nitrate spray.

RESULTS

Effect of successive addition of two quantities of glucose. The possibility that the induction period (apart from nitrogen stimulation) could be ascribed entirely to an unchanging low permeability of the cell membrane to glucose was examined.

A small quantity of glucose (6·67 μmoles) was added to starved cells and the rate of oxygen absorption rose slowly over 123 minutes to a constant rate which continued until the glucose in the medium was exhausted (Moses, 1954); the rate then fell to the endogenous value. A second, similar, amount of glucose was then introduced and the rate of oxygen uptake became constant after only 17 minutes; the final rates of oxygen uptake were the same in both cases (Fig. 1). In another experiment the second quantity of glucose was added at increasingly long intervals after the exhaustion from the medium of the first quantity; there was a consequent gradual lengthening of the second induction period. When about 12 hours had elapsed between the complete metabolism of the first quantity of glucose and the introduction of the second, the length of the second induction period had risen to the original value of the first induction period (Fig. 2).

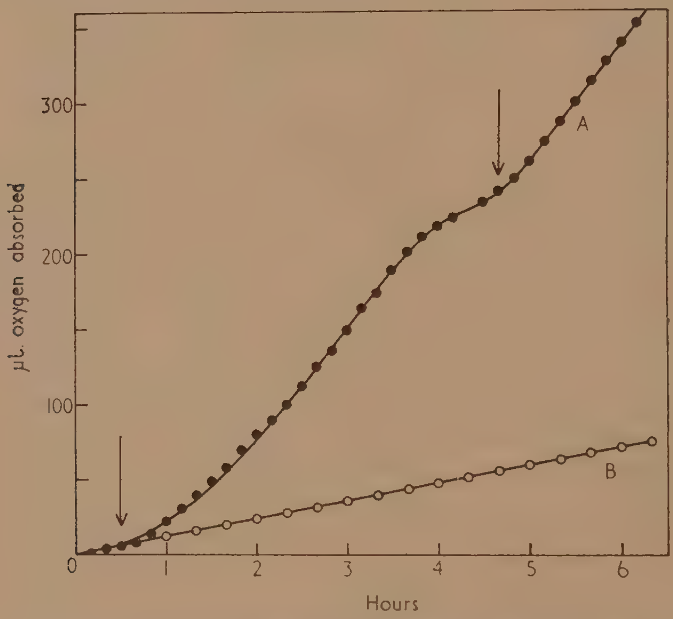

FIG. 1. Addition of two consecutive quantities of glucose to starved cells. A, two consecutive quantities of glucose, each of 6·67 μmoles, added; B, no glucose added. Arrows indicate additions of glucose. Each flask contained 3·7 mg. dry wt. of cells in 0·066 M. phosphate buffer, pH 6·8.

An unchanging low permeability of the cell membrane to glucose would not be sufficient to account for these findings. In that event the induction periods after the addition of the two separate quantities of glucose should have been of equal duration. However, it was possible that the permeability of the membrane to glucose increased in the presence of the substrate. Thus, Deere (1939) advanced an hypothesis of altered membrane permeability of *Escherichia coli-mutabile* in connexion with the oxidation of lactose. Monod (1947) discussed a similar possibility in relation to the nature of enzyme adaptation and pointed out that an increase in a perfect specific permeability to a particular substance would be equivalent to adaptation of a specific 'penetration' enzyme.

The possible effect of permeability on the appearance of the induction period was therefore examined further (*a*) by the use of factors known from other work to affect cell membrane permeability, and (*b*) by investigating the presence of free glucose within the cells after its addition to the external medium.

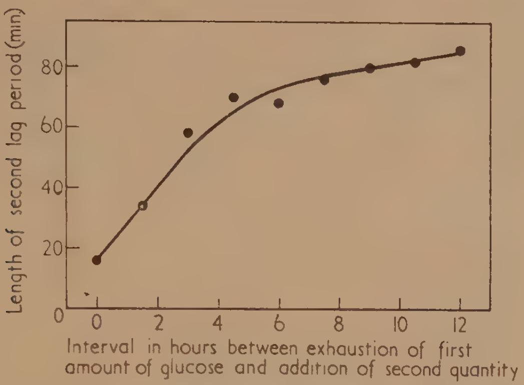

FIG. 2. Increase in the length of the second lag period with increasing intervals between the first and second addition of glucose. The first lag period was 86 minutes in each case. 6·67 μmoles of glucose were added to 4·1 mg. dry wt. of cells at each addition. 0·066 M. phosphate buffer, pH 6·8.

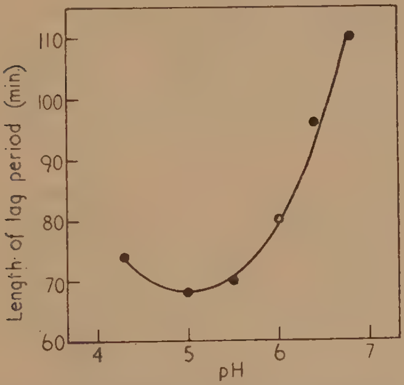

FIG. 3. The effect of pH on the length of the lag period. 66·7 μmoles of glucose were added to 4·4 mg. dry wt. of cells in each flask in 0·066 M. phosphate buffer of the appropriate pH.

Factors affecting cell permeability. Were a low permeability to glucose an important factor in the appearance of the induction period it might prove possible to decrease the length of the induction period by increasing the permeability of the cell membrane. Various factors are known to increase the permeability of some cells towards certain substances and their efficacy in reducing the induction period was tested.

Variations of pH and of the potassium/calcium ratio in the medium did not affect the final rate of oxygen uptake using glucose as substrate. They did, however, affect the length of the induction period.

Examination of the effect of pH on the induction period showed that at an optimum pH of 5·0 the length of the induction period was reduced by about 40 per cent. as compared with that at pH 6·8 (Fig. 3). The latter was the pH normally used. This effect of pH may be due to a permeability change, possibly affecting a system concerned with the entry of glucose into the cells.

The potassium/calcium ratio in the medium affects the permeability of many plant cells (e.g. see Davson and Danielli, 1943). Normally in this work the organism was suspended in potassium phosphate buffer and no calcium was added. When various amounts of calcium were then added and the potassium/calcium ratio thus altered by stages from zero to infinity, it was found that at an optimum ratio of 1 : 10 the induction period was shortened by 30 per cent. as compared with the normal experimental conditions (Fig. 4).

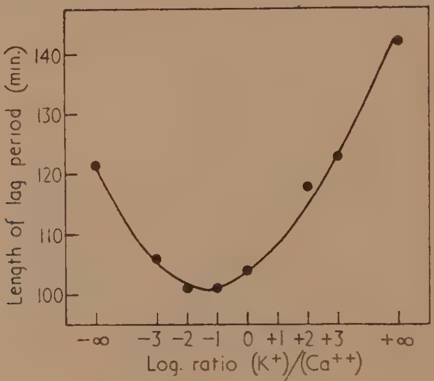

FIG. 4. Effect on the length of the lag period of altering the potassium/calcium ratio in the medium. 66·7 μmoles of glucose were added to 3·6 mg. dry wt. of cells in each flask in a medium containing potassium chloride and calcium chloride in varying proportions.

Several workers (Hotchkiss, 1944; Gale and Taylor, 1947; Salton and Alexander, 1950) have shown that in low concentrations cationic detergents, such as cetyltrimethylammonium bromide, &c., can cause the release of certain intra-cellular constituents from streptococci and staphylococci, this being apparently due to some alteration in the cell membrane resulting in an increased permeability. ‘Cetavlon’ (supplied by Imperial Chemical (Pharmaceuticals) Ltd.), described as ‘a synthetic quaternary ammonium compound containing at least 80 per cent. of trimethyl-(long chain alkyl) ammonium bromides calculated as $C_{16}H_{33}(CH_3)_3NBr$, together with inorganic salts’, was used as a cationic surface-active agent. In high concentrations cetavlon was toxic to the cells, as indicated by a considerable fall in the rate of oxygen uptake in the presence of glucose. However, with sufficiently low concentrations no such toxicity was observed. The maximum concentration of cetavlon showing no toxic effect varied with the particular cell preparation used, and seemed to be roughly proportional to the amount of cellular material present; it was usually 10–12 μg. of cetavlon per ml. When glucose was added to starved cells in the presence of cetavlon the length of the induction period could be reduced by a maximum of about 40 per cent. without reducing the rate of oxygen absorption (Table I).

TABLE I

Effect of cetavlon on the length of the lag period on the addition of glucose. 66·7 μmoles of glucose were added to 3·3 mg. dry wt. of cells/flask in 0·066 M. phosphate buffer, pH 6·8, in the presence of various concentrations of cetavlon

Cetavlon concn. (μg./ml.)	Lag period (min.)	Final constant rate of oxygen uptake (μl./hr./flask)
0	140	180·5
10	86	178·5
15	130	171·9
20	More than 250*	More than 69·6*
25	More than 250*	More than 34·0*
30	More than 250*	More than 29·6*

* At concentrations greater than 15 μg./ml. cetavlon inhibited the rate of oxygen uptake, which increased very slowly when glucose was added. The rate had not become constant by the time the experiment was terminated.

The fact that the length of the induction period could be reduced by techniques known to affect cell permeability made it possible that permeability was responsible at least to some extent for the induction period.

The use of cell-free extracts. Theoretically, a means of eliminating the effects of permeability would be to add glucose to a cell-free extract of the fungus which was capable of oxidizing glucose at a rate comparable with that of whole cells. Numerous attempts to do this by disrupting the cells by grinding them by hand in a mortar with carborundum, glass powder, or sand, grinding them between two rapidly moving, closely opposed glass surfaces (in a tissue homogenizer), by drying them *in vacuo*, and by disruption of a cell suspension by forcing it at high pressure (obtained with a hydraulic press) through a small orifice (Milner, Lawrence, and French, 1950; French and Milner, 1951), have not yielded preparations capable of oxidizing glucose at an appreciable rate, even after the addition of numerous cofactors. No information could therefore be obtained on the influence of permeability on the induction period by this means.

Monod (1947) noted that cell-free extracts of micro-organisms have repeatedly failed to exert any oxidative capacity towards the sugars, though since then a cell-free extract of *Aerobacter aerogenes* has been prepared which oxidized glucose, glucose 6-phosphate, and hexose diphosphate at similar rates (Tissières, 1952).

Investigation of the rate of entry of glucose into the cells. The significance of permeability was further investigated by examining directly the concentration of free glucose inside the cells at intervals after the sugar had been added to the external medium.

A suspension of starved cells in buffer was prepared in the usual way, but with the concentration of the cellular material four times greater than normal (i.e. about 8 mg. dry weight of cells per ml.). A portion (0·5 ml.) of this suspension was pipetted into each of four Warburg flasks for respiration measurements, and 17·5 ml. were placed in a 100-ml. Erlenmeyer flask which was also shaken in the Warburg water-bath. At the appropriate time 0·25 ml. of a 4 per cent. (w/v) solution of glucose was added to the cells from the side-arms of two of the Warburg flasks, buffer being added in the other two for measurement of the endogenous respiration. Simultaneously, 8·75 ml. of the same glucose solution (containing 1,944 μmoles) were added to the Erlenmeyer flask.

For determinations of glucose in the external medium in the Erlenmeyer flask 1·5 ml. of the suspension were removed at intervals after the addition of glucose, centrifuged rapidly, and 0·5 ml. of the supernatant removed. This was subsequently made up to 10 ml. with water, and two 3-ml. samples were used for glucose determinations. The total glucose in the suspension was found by pipetting 0·5 ml. of the suspension directly into 3·5 ml. of an 86 per cent. (v/v) ethanol solution at 80° and was kept at this temperature for 5 minutes. The suspension in ethanol was then centrifuged and the supernatant removed. The residue was extracted overnight at 25° with 2 ml. of a 60 per cent. (v/v) ethanol solution, centrifuged, and the supernatant combined with the previous one; the pooled supernatants were diluted to 10 ml. with water. Two 2·5-ml. samples were used for glucose determinations.

The difference between the total and the extra-cellular free glucose was assumed to be due to free intra-cellular glucose. The values for glucose assimilated were deduced assuming (*a*) that all glucose not accounted for as reducing sugar or by oxidation had been assimilated, and (*b*) that the original endogenous respiration continued in the presence of glucose oxidation (see the next section).

In order to obtain significant experimental differences by the technique used between the total and the extra-cellular glucose, and hence, by difference, arrive at a value for the intra-cellular concentration, it was necessary to use a very dense cell suspension and a high initial glucose concentration in the medium. The cell suspension was four times as concentrated as that normally used for respiration measurements in the Warburg apparatus, and the initial glucose concentration was 0·074 M. instead of the customary 0·022 M. Attempts to use the usual, lower, concentrations were not successful as the experimental differences to be expected were on the extreme limits of the method of estimation.

The volume of the cells was determined by finding the concentration of glucose in the external medium immediately after a known quantity had been added to the suspension, and assuming that none had entered the cells in the short time required for sampling, but that it was entirely confined to the external medium. The amount of glucose added was 350·0 mg.; analysis of a sample taken immediately after the glucose was added gave the total glucose present as 353·6 mg. Analysis of the supernatant after immediate centrifugation showed that were the glucose distributed evenly throughout the suspension, both inside and outside the cells, the total amount of glucose in the suspension was apparently 387·3 mg. As it was assumed that no glucose had entered the cells, the volume of the cells was calculated as $100-(350{\cdot}0/387{\cdot}3\times100) = 9{\cdot}63$ per cent. (v/v) of the total suspension.

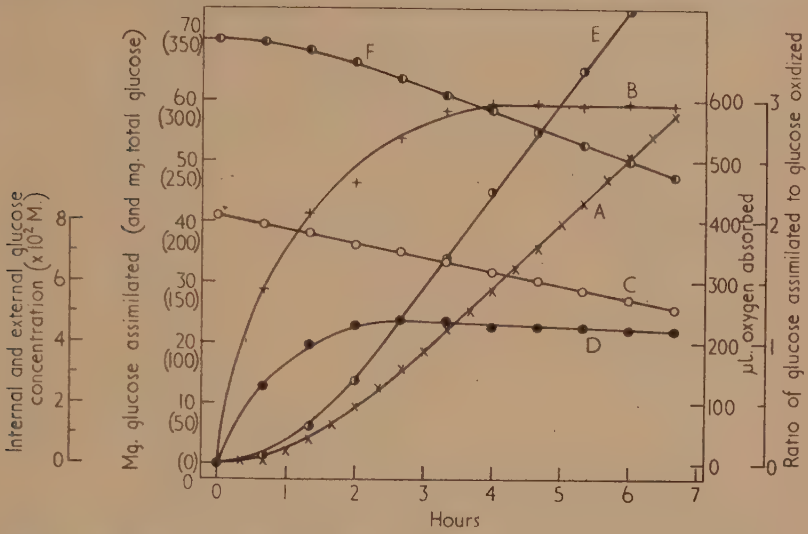

FIG. 5. The entry of glucose into the cells. Initial glucose concentration in the medium was $8{\cdot}19\times10^{-2}$ M. A, oxygen consumed (μl.) after subtraction of the endogenous respiration; B, ratio of glucose presumed assimilated: glucose oxidized; C, concentration of glucose in the external medium ($\times10^2$ M.); D, concentration of intra-cellular glucose ($\times10^2$ M.); E, glucose presumed assimilated (mg.); F, total glucose in the suspension (mg.). Glucose was added to the medium at time 0. For further details see text.

In Fig. 5 the intra- and extra-cellular glucose concentrations are compared with the oxygen consumption after deduction of the endogenous respiration. The amount of glucose assimilated, the ratio of glucose assimilated to glucose oxidized, and the total glucose utilized are also shown. It will be noted that the glucose concentration within the cells never exceeded that in the external medium. Fig. 6 shows the rates of oxygen uptake in the presence of various initial external glucose concentrations, and it is clear that the enzyme system responsible for glucose oxidation was saturated when the external concentration of glucose was $1{\cdot}15\times10^{-2}$ M. The internal glucose concentration necessary for enzyme saturation would not, from the results shown in Fig. 5, have exceeded this value.

In the experiment shown in Fig. 5 the glucose concentration within the cells reached the enzyme saturation level of $1{\cdot}15\times10^{-2}$ M. about 16 minutes after glucose was added to the suspension, so that the internal glucose concentration should not have been limiting for the oxidation rate for longer than this period. Nevertheless, the rate of oxygen consumption did not become constant until about 150 minutes after the glucose was added. Thus only to a small extent could the lag period be ascribed to the time required for glucose to enter into the cells.

The finding that permeability was not the main factor responsible for the lag period rendered superfluous the suggestion that the permeability might change in the presence of the substrate. It does seem likely, however, that when two consecutive quantities of glucose were added (Fig. 1), the small lag period observed after the second addition might be due entirely to the time taken for glucose to enter the cells.

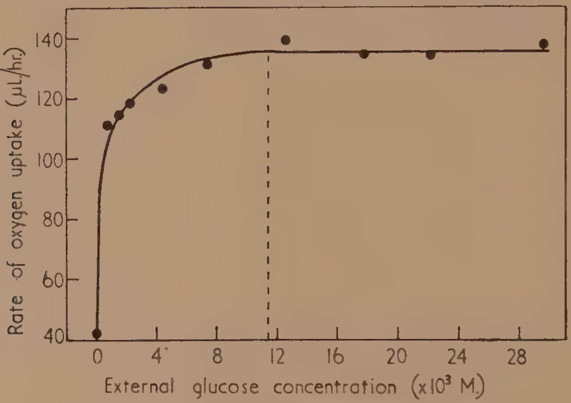

FIG. 6. Effect of the initial external glucose concentration on the final constant rate of oxygen uptake. The endogenous respiration has not been deducted; the rate of oxygen uptake in the absence of added substrate is therefore not zero. Each flask contained 2·8 mg. dry wt. of cells suspended in 0·066 M. phosphate buffer, pH 6·8.

In Fig. 5 the initial external glucose concentration was $8{\cdot}19 \times 10^{-2}$ M., and the glucose concentration limited the rate of oxygen uptake for not more than about 11 per cent. of the total lag period. With the more dilute glucose solutions normally used for respiration experiments the initial external glucose concentration was $2{\cdot}22 \times 10^{-2}$ M. It might have been expected that with a smaller initial concentration gradient of glucose across the cell membrane the time required for glucose to enter the cells and saturate the oxidation system may have been longer, resulting in a longer lag period. In practice, however, it was not possible to reduce the total length of the lag period by increasing the initial external glucose concentration over a fivefold range (Table II). From this it may be inferred that even at the lowest external concentration used in Table II glucose was able to enter the cells and saturate the enzyme system involved in glucose oxidation as rapidly as it could at the higher concentrations.

The continuance of the endogenous respiration during glucose oxidation. The endogenous respiration of *Z. moelleri* is high even after starvation in buffer for 24 hours. From 31 observations in which the endogenous rate of oxygen uptake ranged from 6·8 to 36·6 per cent. of the oxygen consumption in the presence of glucose (depending on the degree of starvation), the average rate of oxygen uptake endogenously was 20·8 per cent. of that in the presence of glucose. Stout and Koffler (1951), and Blumenthal, Koffler, and Goldschmidt (1951) working with *Penicillium chrysogenum*, Cochrane and Gibbs (1951) with *Streptomyces coelicolor*, and Mickelson (1950) with *Ashbya gossypii*, have found that all these organisms have high rates of endogenous respiration.

TABLE II

Effect of various initial external glucose concentrations on the length of the lag period. Each flask contained 4·6 mg. dry wt. of cells in 0·066 M. phosphate buffer, pH 6·8

Initial external glucose concn. ($\times 10^2$ M.)	Lag period (min.)	Final rate of oxygen uptake (µl./hr./flask)
1·110	160	240
1·665	160	243
2·220	150	239
2·775	148	242
3·330	150	250
3·885	149	248
4·440	144	246
4·995	150	250
5·551	150	253

All these authors (except Mickelson, who does not mention this point) conclude that the endogenous respiration of these organisms is not suppressed when they are treated with a respirable substrate. However, Mickelson and Schuler (1953) conclude that the endogenous respiration of *A. gossypii* continues in the presence of acetate as substrate. Gibbs and Wood (1952) came to a similar conclusion with *Pseudomonas fluorescens* as did Norris, Campbell, and Ney (1949) with *P. aeruginosa*. On the other hand, Doudoroff (1940), Wiame and Doudoroff (1951), and Winzler (1940) came to the opposite conclusion from results with *P. saccharophila* and bakers' yeast.

Winzler (1940) proposed as a test for the presence or absence of endogenous respiration in the presence of added substrate the constancy of the ratio of oxygen taken up (with and without correction for endogenous respiration) to the quantity of substrate added. This test was applied to *Z. moelleri*. Small amounts of glucose were added so that the rate of oxygen uptake increased and then returned to the endogenous level as shown in the first half of curve *A* in Fig. 1. The total extra oxygen uptake produced by various small quantities of glucose was measured and Winzler's test applied (Table III): it appears that the presence of at least small amounts of glucose did not suppress the endogenous respiration.

The mechanism of glucose uptake. Several workers have found that the uptake of sugars by the cells of various organisms is connected with an initial phosphorylation at the cell surface. Wilkes and Palmer (1932) found that the region of the living yeast cell in which invertase exerts its physiological activity changes its pH freely, and to the same extent as that of the suspending medium. They suggested that this may indicate that invertase is located somewhere in the outer region of the cell. Rothstein *et al.* (Rothstein and Larrabee, 1948; Rothstein, Frenkel, and Larrabee, 1948; Rothstein, Meier, and Hurwitz, 1951; Hurwitz and Rothstein, 1951) have shown that yeast takes up glucose from the medium by a mechanism involving phosphorylation of the hexose at the cell surface, and suggested that this is the first stage of sugar metabolism by this organism. Dormer and Street (1949) associated sucrose uptake by tomato roots with a phosphorylation at the cell surface. The effect of pH on the lag period of *Z. moelleri* might be related to an effect on surface enzymes concerned with the absorption of glucose into the cells.

TABLE III

The test of Winzler (1940) for the presence of the endogenous respiration applied to glucose oxidation in Z moelleri. For explanation see text

Glucose added (μmoles)	Total O_2 taken up* (μl.)	Endogenous O_2 uptake during similar period (μl.)	Difference between total O_2 uptake and endogenous O_2 uptake (μl.)	μl. O_2 per μmole glucose without subtraction of endogenous respiration	μl. O_2 per μmole glucose with correction for endogenous respiration
0·44	28·5	18·5	10·0	64·1	22·5
1·11	54·0	27·0	27·0	48·6	24·3
1·67	74·5	35·5	39·0	44·7	23·4
2·22	95·5	40·5	55·0	43·0	24·8
6·67	213·5	65·5	148·0	32·0	22·5

* The total oxygen taken up due to the addition of glucose was measured at the time when the increased rate of oxygen uptake following the addition of glucose returned to the endogenous level.

Average oxygen consumption per μmole of glucose added (after correction for the endogenous respiration) = 23·4 μl. = 1·045 μmoles. For total oxidation 6 μmoles of oxygen are required per μmole of glucose. Thus the degree of total oxidation = 17·4 per cent.

Lefevre (1948) showed that the entry of glucose into human red cells was probably via an enzymic phosphorylation involving a sulphydryl group at the cell surface, and the process was blocked by phloridzin. Lundsgaard (1933) also found that glucose was absorbed from the mammalian intestine by an 'active' process, which could be inhibited apparently specifically by phloridzin; the absorption of amino-acids was not affected by this substance.

An attempt was made to investigate the mechanism of glucose uptake in *Z. moelleri* by the use of phloridzin. Only a very slight inhibition of glucose uptake, as measured by the final rate of oxygen uptake, was observed, even when the medium was saturated with phloridzin. However, as only very slight inhibition was obtained, also with saturated phloridzin, on bakers' yeast as well, this organism being known to possess a surface phosphorylase, no definite conclusion could be drawn as to the nature of glucose absorption in *Z. moelleri.*

DISCUSSION

The results presented in this paper show that the permeability of the cell membrane to glucose contributes to some extent to the development of the lag period for glucose oxidation in starved *Z. moelleri*. Techniques known to increase cellular permeability (use of cetavlon, and adjustment of pH and of the potassium/calcium ratio in the medium) were able to reduce the lag period by 30–40 per cent. although investigation of the entry of glucose into the cells indicated that a much lower fraction (about 11 per cent.) of the lag period could be ascribed to limitations imposed by the internal glucose concentration.

A tentative conclusion with regard to the mechanism of glucose uptake might be drawn from the discrepancy observed between the results given in Table II and those found with cetavlon, pH, and changes in the potassium/calcium ratio. Table II showed that an increase in the initial glucose concentration in the medium did not reduce the length of the lag period even though there was an increased initial concentration gradient of glucose across the cell membrane, in spite of the fact that permeability has been shown to play some part in the appearance of the lag period. This would suggest that glucose entered the cells by an 'active' enzymic mechanism which was saturated with glucose even with the lowest initial glucose concentration used in Table II. Cetavlon, &c., might be able to open up or stimulate additional pathways for the entry of glucose into the cells. In addition they might affect the surface and sub-surface architecture in such a way as to enable glucose not merely to enter the cells more rapidly, but also to become metabolized more quickly, and so produce a shortening of the lag period. With the present evidence available, however, these suggestions can be no more than very tentative.

In an earlier paper (Moses, 1954) it was shown that stimulation of glucose respiration by nitrogenous substances in the cells also contributed to the lag period. Nevertheless, most of the lag period cannot be ascribed to either of these two factors, and a further examination of the problem is necessary.

I am greatly indebted to Professor W. H. Pearsall, F.R.S., Mr. P. J. Syrett, and Mr. H. Tristram for their suggestions and criticism, and to the Department of Scientific and Industrial Research for a maintenance grant.

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Physiological Studies in the Mucorales

III. THE ZYGOTROPISM OF ZYGOPHORES OF *MUCOR MUCEDO* BREFELD

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WITH PLATE

Received 28 September 1954

SUMMARY

Some previous work on zygotropism in *Mucor* is briefly reviewed, and further observations confirming earlier studies presented.

In addition to positive zygotropism between (+) and (−) zygophores, there is claimed to be a corresponding 'repulsion' between zygophores of the same mating type (+) from (+) and (−) from (−). The stimulus appears to be transmitted by the diffusion of a volatile substance from one zygophore to another. A tentative explanation of the phenomena described is advanced, based on two postulated volatile hormones, each evolved by zygophores of one mating type only, stimulating growth in zygophores of its own mating type and retarding it in those of the opposite type.

INTRODUCTION

VARIOUS aspects of the process of conjugation in Mucoraceae have in recent years been reviewed in publications by Raper (1952), Goodwin (1952), Hawker (1951), and Catcheside (1951), and briefly discussed by the present author (1954), so no extensive account of the literature need be undertaken here.

There is good evidence for supposing that zygophores of *Mucor mucedo* arise from the vegetative mycelium as a response to a hormone diffusing through the substrate from neighbouring mycelium of the opposite mating type. This paper deals only with the behaviour of these developing zygophores prior to the beginning of conjugation, and is chiefly concerned with their mutual interaction.

In his classical paper announcing the discovery of heterothallism in the Mucoraceae, Blakeslee (1904) observed that 'progametangia arise as the result of the stimulus of contact between (+) and (—) zygophores which in some cases at least have been shown to be mutually attractive and may therefore be termed zygotactic'. Blakeslee illustrated stages in this mutual response, but did not subsequently investigate it in detail. His observations were confirmed and extended by, among others, Burgeff (1924), who introduced the generally

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[1] The work described here was carried out in the Botany Departments of King's College, University of London, and University College, Ibadan, Nigeria. From January 1955 the author's address is the Botany Department, Durham Colleges, University of Durham.

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accepted term 'zygotropism' for the process, which he supposed to be due to a chemotropic influence, and by F. Köhler (1935), who tentatively postulated 'some kind of mitogenic ray'. It is curious that writers of monographs and textbooks have often been unwilling to recognize this zygotropic response, and either ignore the matter or take instead the view expressed by Joyet-Lavergne (1931) that 'it is the *accidental* contact between hyphae of opposite strains that initiates the sexual process leading to zygospore formation'. The whole problem of the mechanism of telemorphic and zygotropic reactions of fungal hyphae in 'sexual' and vegetative fusions is little understood despite the extensive researches of Laibach (1928), E. Köhler (1930), and other workers whose contributions were discussed by Buller (1933) along with his own studies. Buller was impressed by the newly announced claim by Vandendries and Brodie (1933) that the *repulsion* over distances of up to 3·5 mm. observed between certain haplonts of *Lenzites betulina* was transmitted through thin air-tight (? closed) barriers of glass, mica, celluloid, and silver, but not aluminium, copper, platinum, or lead. They concluded that this 'barrage sexuel', which they also observed in *Pleurotus columbinus*, must be due not to a chemical stimulus but to radiations. Buller suggested that 'it may well be that, in the fungi generally, the mutual attraction shown by two hyphae about to fuse with one another is also due to radiation'. Shortly after this Vandendries (1934) withdrew their claim, since he found that when the barriers were truly air-tight no stimulus was transmitted, but among other writers Langeron (1952) fully quotes and discusses their work on the 'barrage sexuel' without being aware of this, and some of the experiments to be described were performed to test a 'radiation' hypothesis before the writer's attention was kindly drawn by Dr. J. H. Burnett to this correction. There is an extensive earlier study by Schmidt (1925) of inhibition zones between vegetative mycelia of Phycomycetes of the same mating type growing on the same substrate. Cayley (1931) tentatively attributed the aversion between aerial hyphae of incompatible monospore mycelia of *Diaporthe perniciosa*, the genetics of which she investigated, to a reaction between a substance, possibly volatile, produced by one mycelium and another complementary substance produced by the other.

In the absence of a clear understanding of telemorphosis and zygotropism it seems worth while to describe in some detail a series of simple experiments designed to elucidate one aspect of these phenomena.

EXPERIMENTAL PROCEDURES

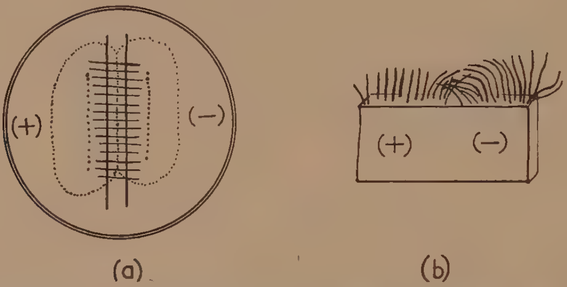
Various (+) and (—) strains of *Mucor mucedo* Brefeld were used in the early stages of this work, but for the later critical experiments strains from the Baarn Centraalbureau voor Schimmelcultures were employed, because they showed most clearly a dimorphism between (+) and (—) zygophores. The (—) zygophores differed from the (+) in being richly provided with orange-yellow carotene near the tip and also in being mostly longer and slimmer, and therefore they could usually be identified in a tangle containing both. Paired colonies of opposite mating type were grown to form copulation zones on

2 per cent. agar containing 3 per cent. malt extract and 0·5 per cent. peptone. This addition of peptone seemed somewhat to delay the development of sporangiophores and sterile aerial hyphae behind the edge of the colony, so leaving for a time unobscured the zone where the zygophores appeared when the colonies grew into juxtaposition. The peptone supplement also favoured rich development of carotene in the (—) zygophores. (It has often been claimed that on the average (+) strains tend to have more carotene in their mycelium than (—) strains of the same species: whether this is so is uncertain, but in any case the (—) zygophores of the strains used in this work had a characteristic high content of carotene. In chromatographic separations this carotene behaved like a sample of β-carotene, and not like γ-carotene extracted from male cells of *Allomyces javanicus*.)

The agar plates were inoculated with alternate transverse streaks of suspensions of (+) and of (—) spores in sterile water. If the streaks were parallel, the whole length of the copulation zone reached approximately the same stage at the same time; if not, stages from early zygophore initiation to zygospore maturation could be found as one proceeded along the zone of contact in the direction of convergence of the inoculation streaks. Viewed from above the zygophores presented a clear picture of curvature as if mutually attracted, but it seemed possible that such an impression might be given if the aerial zygophores had an inherent tendency to turn over, and then to grow centrifugally away from the older part of the colony as do the main hyphae on the agar surface. It therefore seemed desirable to isolate small numbers of zygophores together with sufficient supporting substrate and to examine their behaviour more critically.

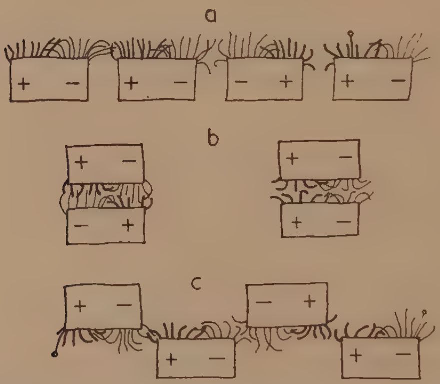
Unfortunately, despite various attempts (see Raper (1952) for references, and Banbury (1954)), no reliably reproducible method has yet been developed for obtaining a hormone preparation which will give a good development of zygophores of one mating type in the absence of the other. Also young zygophores, when excised from the main substrate through which such a hormone is presumably diffusing to them from mycelium of the opposite mating type, develop into sporangiophores with terminal sporangia. Observations were therefore mostly made using small blocks of agar (e.g. 1·5 mm. wide × 3·5 mm. long on the upper surface × 3 mm. deep) which were cut transversely from the copulation zone between (+) and (—) colonies newly grown into contact in a Petri dish culture prepared as above (Text-fig. 1*a*). In this way one obtains a tuft of unmixed (+) zygophores at one end of the block, a mixed tangle of copulating hyphae in the middle, and (—) zygophores at the other end (Text-fig. 1*b*). Zygophores, unlike sporangiophores, do not show geo- and phototropic responses, so it was usually convenient to turn these blocks over on to their side, so that the zygophores then elongated in horizontal rather than vertical planes, and were more easily examined, drawn, and photographed while growing undisturbed in a moist chamber under the microscope.

The microscope slides bearing these rows of blocks were then incubated in a moist chamber at 18–22° C., and examined at intervals after 8 and up to 18 hours. As zygophores soon wilt in dry air, it was found necessary when observing the results to transfer the slides in turn to a Petri dish base containing a wet paper pad and provided with a flat sliding top of thin perspex. Through this lid a central hole had been cut just large enough to admit the body of the 4-mm. objective of the microscope, which could slide down like a piston till the zygophores were in focus.



Text-fig. 1. (*a*). Diagram to show method of isolating blocks. A Petri dish is shown and the positions of vertical incisions indicated by continuous lines. The dotted lines show the extents of the (+) and (–) colonies with copulation zone between.

Text-fig. 1. (*b*) shows one of the blocks separated with copulation zone in the middle, on larger scale.



Text-fig. 2. Diagram of results observed with various arrangements of blocks; (*a*) end to end in series, (*b*) opposed in parallel, (*c*) opposite to one another cornerwise. (Diagrams not to scale.)

GENERAL OBSERVATIONS OF ZYGOTROPISM

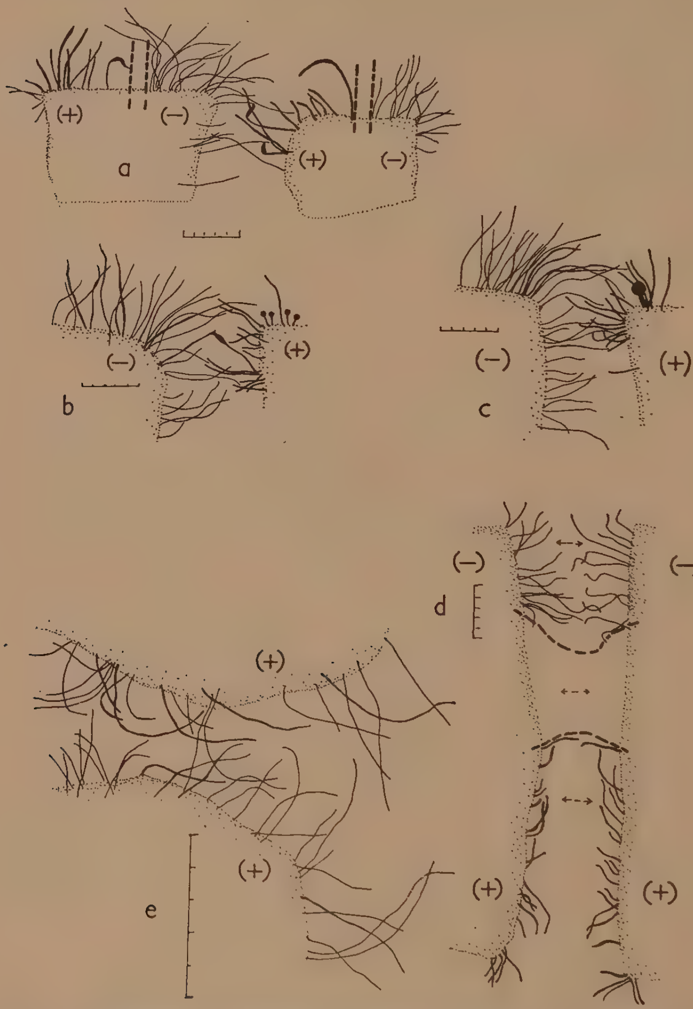
Successive series of these blocks were arranged by hand or micromanipulator so that zygophores of one type were brought close to those of either the same or alternatively the opposite mating type arising from a neighbouring block, pointing in various directions and at various distances (some arrangements are illustrated in Text-fig. 2). Many such observations on (+) and (–) zygophores of *Mucor mucedo* have persuaded the writer that there can be no reasonable doubt that there is a mutual zygotropic response between them. Whether they come into proximity growing spontaneously or as a result of experimental manipulation, young (+) zygophores and (—) tend to grow toward one another, if necessary curving in the process. Using a micromanipulator it occasionally proved possible, after selecting a zygophore curving in one direction, to bring the tip of a second zygophore of opposite mating type near to it and to the outside of its curvature, and then at intervals to withdraw it slightly, and so to induce a reverse curvature in the first zygophore. Such attempts often failed because of cessation of growth, or through interference arising from the growth of other neighbouring hyphae, and the results are difficult to illustrate without a cinematographic record, but the experience left the operator strongly impressed with the reality of the positive zygotropic response.

Another significant point which does not seem to have been observed before, but is also evident using the 'paired blocks' technique, is that there is a mutual aversion between zygophores of the same mating type: (+) zygophores are repelled by (+), and (—) by (—). It is clear from the curvatures shown in the photographs (Pl. I) and the drawings (Text-fig. 3), which should be self-explanatory, that the direction of growth of the zygophores is predominantly influenced by neighbouring zygophores. The direction of growth of the hyphae on the substrate had been from the ends towards the centre of the upper surface of the block, but zygophores readily turned back against or across this direction of growth when attracted by zygophores of the opposite mating type brought into their vicinity. The appearance of a row of these blocks is curiously reminiscent of an arrangement of bar magnets, with the zygophores representing the distribution of iron filings along 'lines of force', converging between unlike poles and diverging between like! The attraction has been detected over a distance of 2 mm., and the repulsion is clear at 1 mm. Neither effect was shown when zygophores were confronted by blocks of sterile agar, or blocks on which young substrate mycelium, or vegetative mycelium bearing sporangiophores but no zygophores, was growing. This absence of response was found whichever mating type was used in any combination, even when the distance between the blocks was reduced to a minimum.

THE TRANSMISSION OF THE ZYGOTROPIC STIMULUS

In view of the suggestions of F. Köhler, Vandendries and Brodie, and Buller, it seemed important carefully to test the 'radiation' hypothesis. Tests were first made of the effect of thin barriers erected between blocks bearing tufts of zygophores arranged as in the previous experiments. Thin lamellae of fused quartz, glass, mica, aluminium foil, and waxed cigarette paper were employed, the extremes ranging between 2 and 50 mg./cm.^{2} in density. These barriers, some up to 8 mm. high, were cemented with Durofix or Canada balsam in a vertical position between the long edges of pairs of microscope slides lying side by side on glass plates: the assembly was then sterilized.

Blocks were arranged closely on either side of the barriers and then incubated and examined as above.



TEXT-FIG. 3. Drawings of zygophores growing from the corners of agar blocks prepared as described in the text. All hyphae in the field of view have been drawn except those in the dense tangle of the copulation zone—between the broken lines in (*a*) and (*f*). The depth of field here represented on a surface was 0·2 to 0·3 mm.; the attraction or repulsion was more clearly evident in three dimensions. The scales inserted in the figures are 0·5 mm. long in each case.

(*a*) Attraction between (+) and (−) zygophores on adjacent corners, and normal distribution at isolated corners. (*b*) to (*c*) Various stages illustrating attraction between (+) and (−) zygophores. (*d*) Repulsion between (+) and (+) and between (−) and (−). The blocks were placed in parallel opposite to one another; they were moved 0·2 mm. farther apart to facilitate drawing. (Insert.) (*e*) Repulsion between well-developed (+) and (+) zygophores; blocks arranged end to end.

No genuine attraction or repulsion was ever observed under these conditions; although some initial confusion was caused by reflections from the surface of glass and mica barriers that had slipped slightly out of the vertical, probing with a fine needle eliminated such misinterpretations. Attraction was, however, sometimes detectable when mica or aluminium foil barriers with one fine perforation were used, (+) and (—) zygophores respectively curving from opposite sides and growing towards the pore even in a few cases when the arrangement was such that no straight path existed between any zygophore on one side through the hole to one on the other side. This last observation seems incompatible with any radiation hypothesis. Attempts were made to produce a double-baffle barrier as a confirmatory test. A sheet of aluminium foil folded in two was carefully pricked at close regular intervals using a microscope fitted with a fine needle attached to the objective and a graduated mechanical stage as a 'sewing machine'. The top sheet was carefully slid out of phase by half the length between two holes, and then cemented down at the edge, and the perforated portion was cut out and erected as a double barrier. No zygotropic response was detected, but considering the tortuous and obstructed diffusion path this is not surprising. However, in other experiments a (+) zygophore isolated by bending back its neighbours with a fine needle was brought near to a similarly isolated (—) zygophore, and between them a fine current of moist air was alternately blown from and drawn into a micro-jet, so breaking down normal diffusion between them. In no case did the zygophores curve towards one another, though the experiment was not quite conclusive as they tended after a time to wilt. The writer subsequently learned that a somewhat similar idea had been developed by Kehl (1937), but on a macro-scale; she found that an air current modified the development of the copulation zone, an observation consistent with the above.

DISCUSSION

The above results are considered to demonstrate that zygotropism in *Mucor mucedo* is a form of chemotropism and not a response to any electromagnetic radiation. As suggested by the work of Ronsdorf (1931) on *Phycomyces blakesleeanus* and Krafczyk (1931, 1935) on *Pilobolus crystallinus*, and by Buller (1933) in his discussion, it seems that two substances must be involved in mutual zygotropism. Mating zygophores of *Mucor mucedo* usually meet at or near their tips, as would be expected, since they grow towards one another, and progametangia are not formed on the basal region of zygophores, where the wall may be resistant to renewed stretching. The foregoing explanations may completely account for the structural relationships found at copulation but it also may well be that the terminal rather than the basal portions of zygophores are the main source of these volatile chemotropic growth-regulators. It seems necessary to postulate two of these substances, one evolved by (+) zygophores, stimulating elongation growth in (+) zygophores and retarding it in (—) zygophores, the other evolved by (—), stimulating growth in (—) and retarding it in (+). Then a (−) zygophore lying transverse to a declining concentration gradient from a source of the substance evolved from (+) zygophores would curve towards it, and vice versa with the substance evolved from a (−) zygophore.

The mutual attraction or positive zygotropic response is readily demonstrated; it has been interpreted here as a consequence of the partial inhibition of growth on the near side of each of two hyphae caused by the higher concentration on that side of a growth regulator which, diffusing from the other, has a specific inhibitory action on zygophores of mating type opposite to that of its source.

The outermost zygophores of a tuft have a tendency to curve outwards from the perpendicular direction of growth shown by those towards the centre of the tuft. This is easily interpreted in terms of the postulated growth-promoting action of the volatile emanation of zygophores of one mating type on others of the same type, for those in the centre of a tuft would be surrounded by a uniform concentration of the substance, those at the edges on the fringe would be more stimulated on the side facing inwards and so would curve slightly outwards. This corresponds with the typical picture presented by zygophores at the edge of a block not confronted by another. Repulsion can therefore only be clearly demonstrated when the tips of like zygophores arising from separate blocks and growing in different directions are brought into juxtaposition, so jointly producing in the air between them a concentration of the volatile growth-promoter higher than that on the other side of each of them contributed by the rest of the like zygophores growing on the same block. In such a situation a distinct and otherwise inexplicable change in direction of growth is found.

In discussing the phenomenon of zygotropism the established term 'chemotropism' has been used. Cook and Elvidge (1951) have shown that a number of hydrocarbons, ethers, and esters in dilute solution in sea-water attract free-swimming spermatozoa of *Fucus* spp., and though the mechanism of their action is unexplained, the authors suggest that it may be primarily physical in nature, for the spermatozoa are sensitive only to a concentration gradient of the attractive substance. If this is also true for zygophores, the term 'chemotropism' may not be strictly appropriate for the attractive process.

The diffusion pattern in *Mucor* must be rather complicated if the hypothesis we have proposed is correct. For a point source slowly and constantly evolving a volatile substance the concentration at a point some distance d away will be inversely proportional to d^2 if the process is one of simple diffusion. Regarding the terminal region of a zygophore as approximately a point source, then if another zygophore 10 μ thick lay transverse to the diffusion path at a distance of 2 mm., the concentration of molecules reaching the front face would be about 1 per cent. greater than at the far side of the zygophore. With many neighbouring zygophores forming an extended source the difference in concentration of the active substance across the breadth of the receiving zygophore will be rather less, and it will also be less at greater distances, but for the conditions actually observed it seems unlikely that the difference would fall below 0·3 per cent. It has been estimated (Banbury, 1952) that *Phycomyces* sporangiophores will show a positive phototropic curvature when the light energy absorbed in the rear half of the sensitive region of the sporangiophore is only about 0·2 to 0·5 per cent. greater than the energy absorbed in the front half. It is interesting that these independent estimates give answers of the same order of magnitude for the limiting difference necessary for an observable response to a tropic stimulus by comparable aerial hyphae.

Now it is suggested that when there is a mutual zygotropic response between (+) and (—) zygophores two different volatile growth-regulating substances, with the diffusion gradient of one in a direction opposite to that of the other, must be involved. The further growth of each zygophore will thus be influenced by the concentration gradient of the substance diffusing from the other.

To confirm the conclusions drawn in this paper further work on isolated pairs of zygophores is envisaged, with attempts to measure actual growth rates before and after they are brought into juxtaposition. Attempts are also being made to influence the direction of growth of zygophores by using open capillary pipettes containing extracts of other zygophores or volatile substances of known composition. No firm conclusions can as yet be drawn from the early stages of this work, and further discussion is better postponed till adequate experimental evidence is available.

ACKNOWLEDGEMENTS

I am very grateful to Professor T. A. Bennet-Clark and Dr. G. Metcalfe of King's College, London, for their continued interest throughout the slow progress of this work, and to them and to Professor N. S. Alexander, Professor F. W. and Dr. Eva Sansome, and Mr. R. M. Jackson of this College for their helpful advice and criticisms, and for facilities placed at my disposal. I should also like to thank Dr. J. H. Burnett of Liverpool University for the information and ideas he has given me in our discussions of this subject.

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* This paper is by Franz Köhler, but I have recently received a letter from Dr. E. Köhler emphasizing that he holds different views from F. Köhler and suggesting that there has been some confusion between them in published work, including my own (1954). I hope to clarify the position in a subsequent publication after further correspondence.

DESCRIPTION OF PLATE

(*a*) Attraction between (+) and (−) zygophores growing between blocks placed end to end.

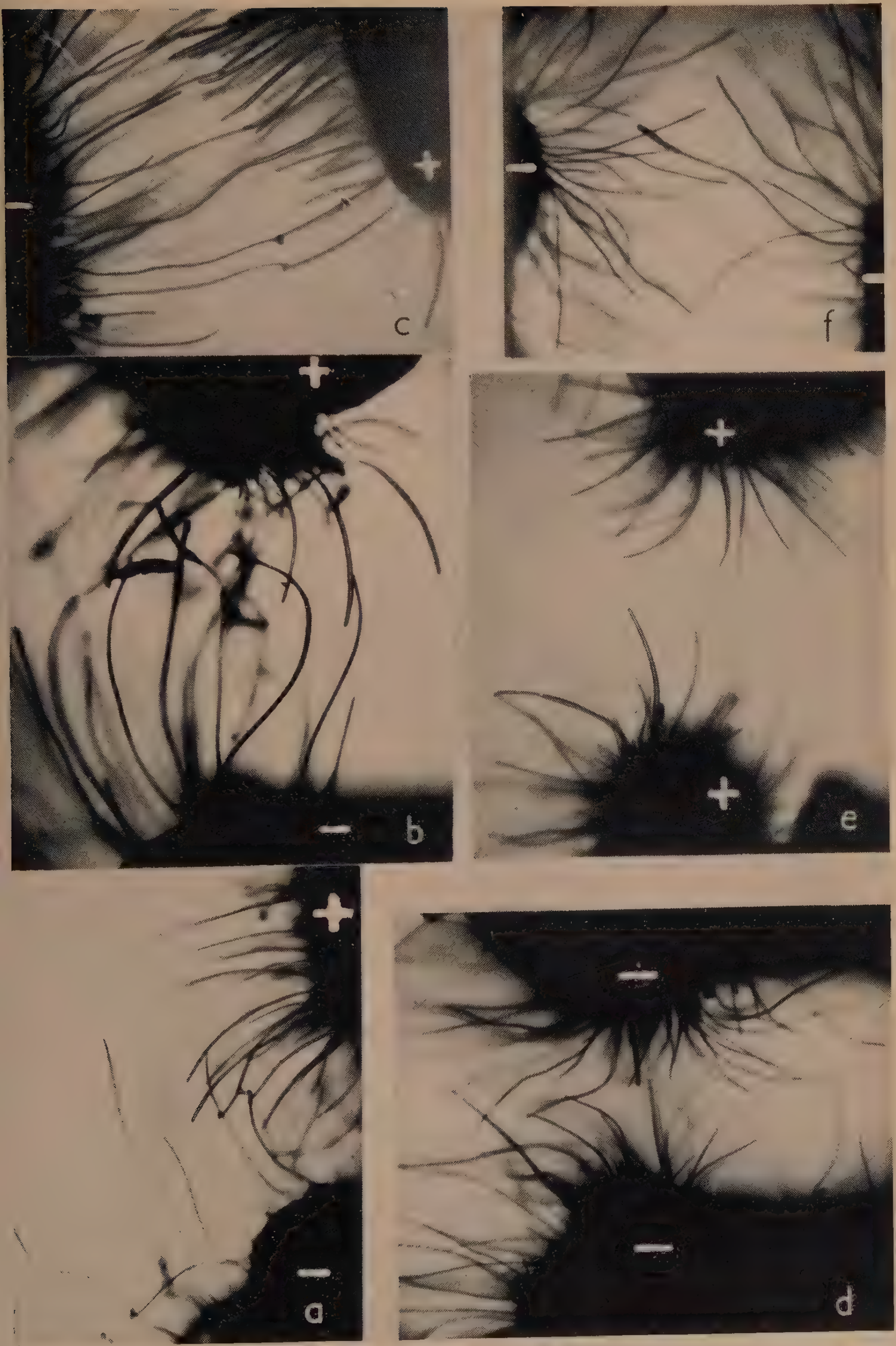
(*b*) Attraction between (+) and (−) zygophores growing between blocks placed opposite one another cornerwise.

(*c*) Attraction between (+) and (−) zygophores growing between blocks placed in parallel opposite one another.

(*d*) Repulsion between (−) and (−) zygophores growing between blocks placed end to end.

(*e*) Repulsion between (+) and (+) zygophores growing between blocks placed opposite one another cornerwise.

(*f*) Repulsion between (−) and (−) zygophores growing between blocks placed in parallel opposite one another.



G. H. BANBURY

The Growth Substances separated from Plant Extracts by Chromatography

II. THE COLEOPTILE AND ROOT ELONGATION PROPERTIES OF THE GROWTH SUBSTANCES IN PLANT EXTRACTS

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SUMMARY

1. A method for the running of 'strip' chromatograms of plant extracts, as large-scale sources of the naturally occurring growth substances accelerator α (α) and inhibitor β (β), and the elution of these substances together with indole-3-acetic acid (IAA), is described. A method is given for the testing of the pea root section extension properties of these growth substances.

2. Coleoptile and root section extension tests over a complete concentration range are done for α, β, and eluted IAA, and mixtures of α and β with IAA or indole-3-acetonitrile (IAN) are tested for coleoptile section extension.

3. α promotes at low concentrations and inhibits at high concentrations both coleoptile and root section extension and the coleoptile section extension induced by IAA or IAN. β inhibits coleoptile and root section extension over the whole concentration range; it also inhibits IAA and IAN induced coleoptile section extension.

4. The extension of coleoptile sections in mixtures of α or β with IAA is measured at a number of time intervals. α, alone and with IAA, has its greatest promoting effect in the early stages and its greatest inhibiting effect in the later stages of section growth. β, alone, promotes the early stages and inhibits the later stages of section growth and, with IAA, has its greatest inhibitory effects in the later stages.

INTRODUCTION

In Part I of this series (Kefford, 1955) the occurrence of the growth substances indole-3-acetic acid (IAA), accelerator α (α), inhibitor β (β), and a substance provisionally described as indole-3-acetonitrile (IAN) in many plant species and in a wide variety of plant tissues was established. This paper deals with some of the properties of these substances, with particular reference to extension growth. Some idea of the effects of the above growth substances upon *Avena* coleoptile section extension was gained from the assay of chromatograms of extracts containing them; but, for a full understanding of their extension properties, the shape of the coleoptile section elongation curves over a wide range of concentrations and the activity of the substances at physiological concentrations should be known. The growth activity of α and β, with respect to roots, was not known at all and it was of particular interest to find if inhibitor β, which inhibited coleoptile section growth, could promote root section growth. To obtain the large amounts of α and β necessary for

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these tests, large-scale chromatograms were developed and the active substances eluted from them. The scale of the chromatography has been enlarged by replacing the spot of extracted material, as used on previous chromatograms in Part I, by a strip and by using a thick chromatographic paper, which could cope with a large amount of extracted material per unit area. It should be emphasized that the above-mentioned identification of occurrence of IAN is somewhat tentative; it is based on identity of spray colour reactions, R_F, and also on a very similar type of biological activity in the *Avena* extension test. It seems likely on the basis of colour reactions that it is an indole-derivative at least.

Foster, McRae, and Bonner (1952) and McRae and Bonner (1952, 1953) applied the enzyme kinetics of Michaelis and Menten and Lineweaver and Burk to the elongation of *Avena* coleoptile sections in added growth substances and mixtures of growth substances. Some synthetic growth substances have been grouped as 'auxin antagonists' and those antagonists with no auxin activity of their own have been called anti-auxins. It was claimed by McRae and Bonner (1952, 1953) that anti-auxins act as competitive inhibitors of the coleoptile extension induced by IAA and some synthetic auxins and they used a Lineweaver and Burk treatment to prove their point. Audus (1954) has provided a critical discussion of some of the problems involved in these interactions. Some of the results of the interaction of IAA with α and β are dealt with here.

MATERIALS AND METHODS

Preparation of eluates containing α or β. The extracts of plant material were prepared and purified as described in Part I (Kefford, 1955), except that larger amounts of material were treated. Extracts containing acidic substances from up to 600 g. of plant material were applied as strips, of lengths increasing to 35 cm., on sheets of No. 1 Whatman paper, 47 cm. × 42 cm. Extracts of more than 600 g. and up to 3,000 g. of material were applied as a strip to a sheet of Whatman 3 MM paper of the same dimensions. With a stream of nitrogen directed along the starting line, the ether extracts containing acidic substances were applied from a dropping pipette as a strip of minimum thickness. On some sheets a small fraction of the extract was applied as a spot to act as a marker chromatogram, while on other sheets a part of the large chromatogram was cut out after development to serve this purpose. The usual IAA marker spot was placed on all sheets.

The chromatographic procedure was exactly as described for small chromatograms in Part I, the solvent always being isopropanol: water: : 10: 1 with ammonia added in the base of the tank. On some chromatograms the solvent was required to run farther than the paper sheet allowed and in these cases extra paper was added as a roll to the base of the sheet. After development of the chromatogram, the paper was dried, the strip holding the IAA marker spot was cut off and sprayed with ferric-perchloric spray (Kefford, loc. cit.), and the extract marker chromatogram was cut into squares and bioassayed with *Avena* coleoptile sections in the usual way. In the meantime the large chromatogram was stored in nitrogen at −10° C. in darkness. The results of the bioassay of the marker chromatogram, with the aid of the IAA marker spot, located the areas on the large chromatogram holding growth-active substances, and strips including these areas were cut from the paper sheet. The active substances were eluted from these strips in two ways. In early experiments the chromatography apparatus was used and absolute alcohol was allowed to flow down the strip and drip from a point at the base into a receiver, carrying the active substance with it. This elution method proved to be inefficient, so, in later experiments, the strips holding growth-active substances were cut into 3 × 3 cm. squares which were boiled in turn with 3 × 10 ml. of ether and 3 × 10 ml. of absolute alcohol. The elutions were continued for a further period with fresh solvents to test their efficiency and the second method was found to be consistently efficient. The eluates which contained accelerator α, inhibitor β, or IAA were obtained as solutions in absolute alcohol; these were stored at −10° in darkness, then, as required, were diluted with alcohol or were concentrated.

The biological testing of eluted substances. For testing with *Avena* coleoptile sections, the eluted α, β, or IAA was added to the test dishes in 1 or 2 ml. of alcoholic solution, then the alcohol was evaporated on a water bath. Three per cent. sucrose solution, 2 ml., was placed in the dishes and an average of 12 *Avena* coleoptile sections were added. The coleoptile extension over 20 hours was measured. When synthetic IAA or IAN were used in the interaction tests with α or β, these substances were added to the test dishes in alcoholic solution from a micropipette.

When α, β, or IAA, eluted from plant extract chromatograms, were to be tested for pea root section extension, they were added in alcoholic solution to 25 ml. conical flasks. The alcohol was removed by heating on a water bath and 2 ml. of 1 per cent. sucrose solution was added to each flask. The pea root sections that were used in these assays were obtained as follows. *Pisum sativum* (var. 'Pilot') seeds were sterilized and soaked in shallow water for 8 hours, then were allowed to germinate on moist paper pulp until the radicles of a majority of seeds just pierced the testa (40 hours from soaking, at 22°). Seeds at this stage were planted, with roots pointing downwards, in moist sawdust watered uniformly from beneath. When the roots were approximately 5 cm. long (110 hours from soaking), those with lengths within a range of 0·5 cm. either side of the average root length were selected. Without allowing the selected roots to dry, the final 1 cm. from each root was placed in a cutter, which removed a tip of 1·5 mm. and took the next 1·5 mm. section, which was placed in the test flask. Twenty sections were added to each of the flasks; these were placed in a water bath held at 25° and were shaken vigorously for 20 hours, when the elongation of the root sections was measured with a microscope fitted with a scale in the eyepiece. This method was not used for the bioassay of squares cut from the chromatograms, since the presence of paper interfered with the growth of the root sections.

EXPERIMENTAL RESULTS

The extension growth properties of α and β eluted from plant extract chromatograms. The unit of concentration used for accelerator α, inhibitor β, and eluted IAA was empirical, normal (N) being the concentration obtained when all of the active substance eluted was dissolved in the same number of ml. as g. of plant material that were extracted to give the substance.

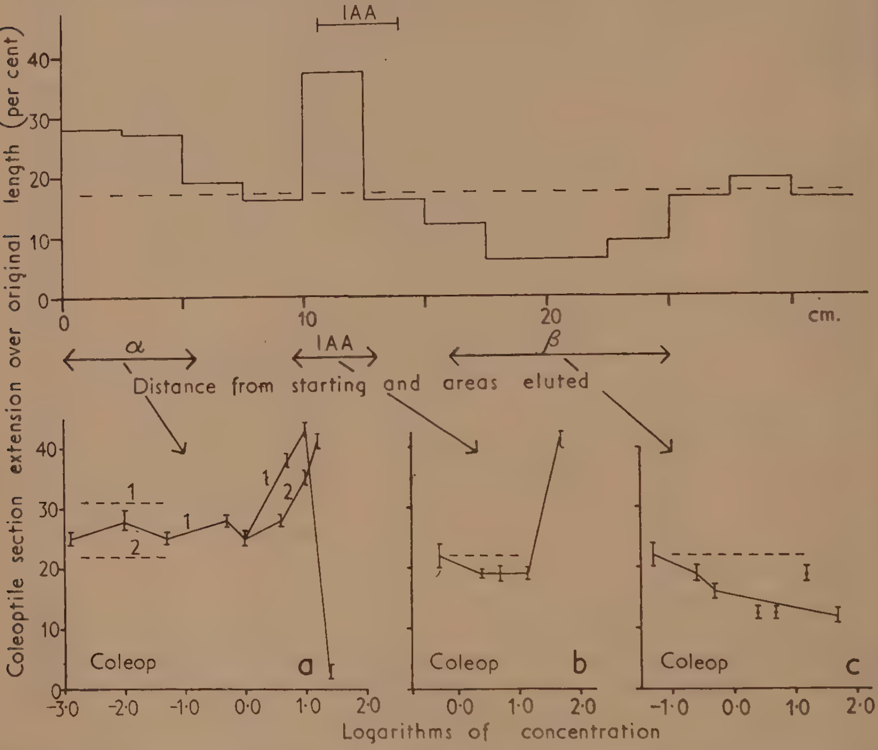

FIG. 1. The activities in *Avena* coleoptile section extension of accelerator α (*a*), IAA (*b*), and inhibitor β (*c*) eluted from areas, on a broad-bean *shoot* extract chromatogram, occurring at distances from the starting line as indicated. Two tests were done with α. The results of tests on the marker chromatograms that located the areas are shown. The broken horizontal lines are extensions of controls. Concentrations of eluates are given as logarithms of normalities as defined in text.

An experiment involving 180 g. of etiolated broad-bean shoots is described diagrammatically in Fig. 1. Extract containing acidic substances corresponding with 30 g. of shoots was used in the marker chromatogram and the results of its bioassay using *Avena* coleoptile sections is given as a histogram, on which the IAA marker position is also shown. The areas of growth activity due to α, IAA, or β on the large chromatogram (extract from 150-g. shoots), which were located from the marker chromatogram and were eluted by boiling with ether and alcohol, are indicated below the histogram by symbols ↔. The effects upon *Avena* coleoptile section extension of the eluted α, β, and

IAA were studied. The results of two tests with series of concentrations of α are graphed in Fig. 1*a*, lines 1 and 2, and those with series of concentrations of IAA or β in Fig. 1*b* and 1*c*. The two tests done with α (Fig. 1*a*) showed this substance could promote coleoptile section growth, but the maximum increase in growth, attainable with increase in concentration, was low when compared with IAA, which at 1·5 mg./l., with the same batch of coleoptiles, gave an increase in extension about three times the maximum with α. Concentrations of α above the optimal for growth were strongly inhibitory; in fact the whole growth effect of this substance upon coleoptiles appeared to take place within a narrow range of concentrations. Similar results were obtained with an α eluate from pea shoot extract (Fig. 3*a*). The inhibitor β eluate (Fig. 1*c*) was found to inhibit coleoptile extension at all concentrations and another demonstration of this is seen in Fig. 3*d*. The concentration *v.* growth curve for eluted IAA (Fig. 1*b*) was typical for suboptimal concentrations and a curve for a full concentration range, obtained with IAA from an etiolated potato shoot extract, is shown in Fig. 3*b*.

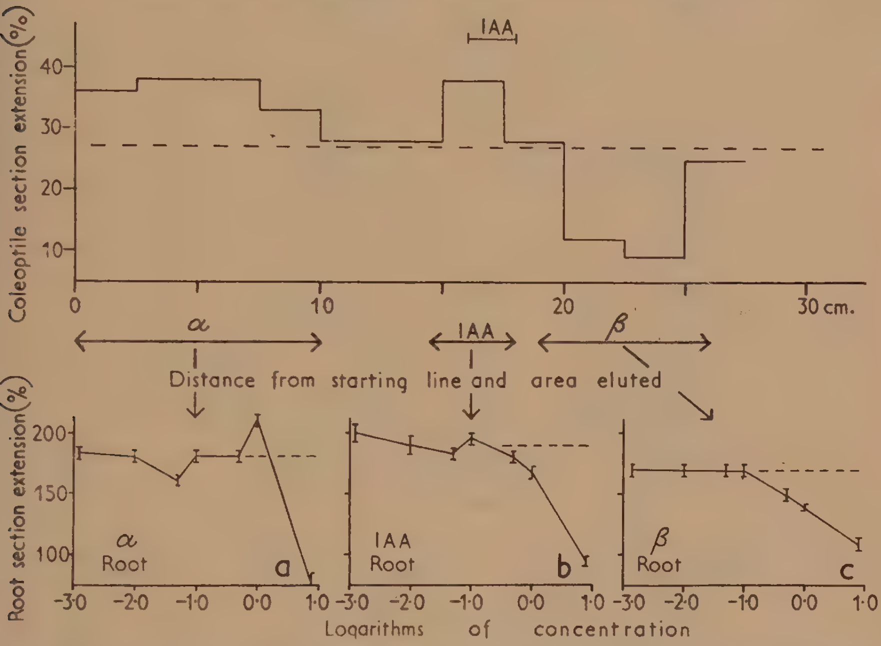

FIG. 2. The activities in pea root section extension of accelerator α (*a*), IAA (*b*), and inhibitor β (*c*) eluted from areas, on a broad-bean *root* extract chromatogram, occurring at distances from the starting line as indicated. The results of tests on the marker chromatograms, that located the areas, are shown. Conventions as in Fig. 1.

In Fig. 2 the pea root section extension properties of α, IAA, and β, eluted from a broad-bean root extract chromatogram, are demonstrated as in Fig. 1. From broad-bean roots, 4 cm. long, the first 1·5 cm. section from the tip was removed to yield 32 g. material. Of the extract containing the acidic substances, one-third was used for the marker chromatogram and the remainder for the chromatogram to be eluted. The histogram of the results of the bioassay of the marker chromatogram with *Avena* coleoptile sections, the IAA marker spot, and the limits of the areas eluted are indicated on Fig. 2. Series of concentrations of eluted α, IAA, or β were tested for pea root section extension and these results are shown in Fig. 2*a*, *b*, and *c*. The tests were not done with the same batch of roots so are not strictly comparable. One concentration of α promoted the growth of pea root sections (Fig. 2*a*), but higher concentrations were inhibitory. The root growth promoting property of α was seen more clearly with eluates of α from pea shoot extract (Fig. 3*c*) or from potato shoot extract (Fig. 3*e*). The eluted IAA gave a root growth *v.* concentration curve (Fig. 2*b*) typical of a growth inhibitor. As with coleoptile section extension, β proved to be an inhibitor of root section extension at all concentrations (Fig. 2*c*).

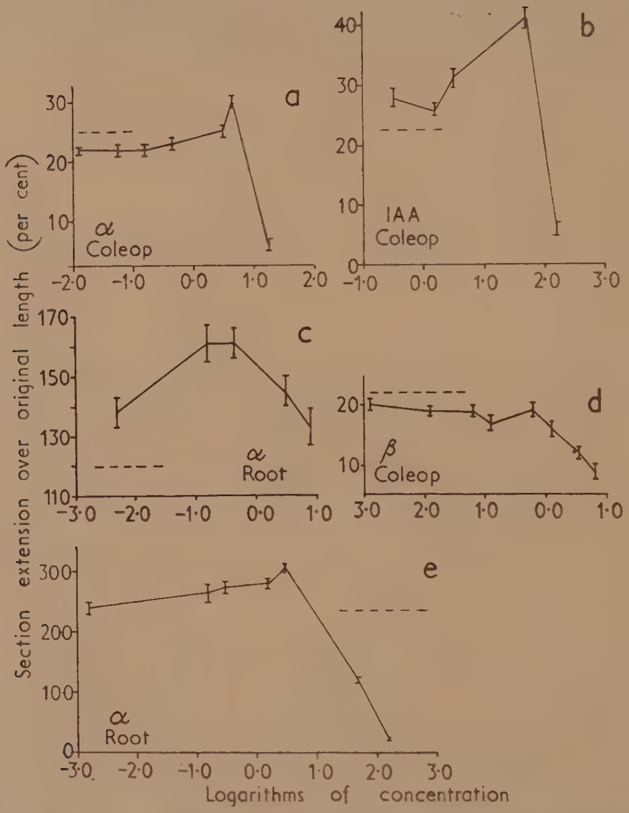

FIG. 3. The activities in root or coleoptile section extension of accelerator α, IAA, and inhibitor β. The vertical lines are twice the standard errors.

Some further experiments have been done in which the interaction of these substances in *Avena* coleoptile extension was studied. As before, α and β were

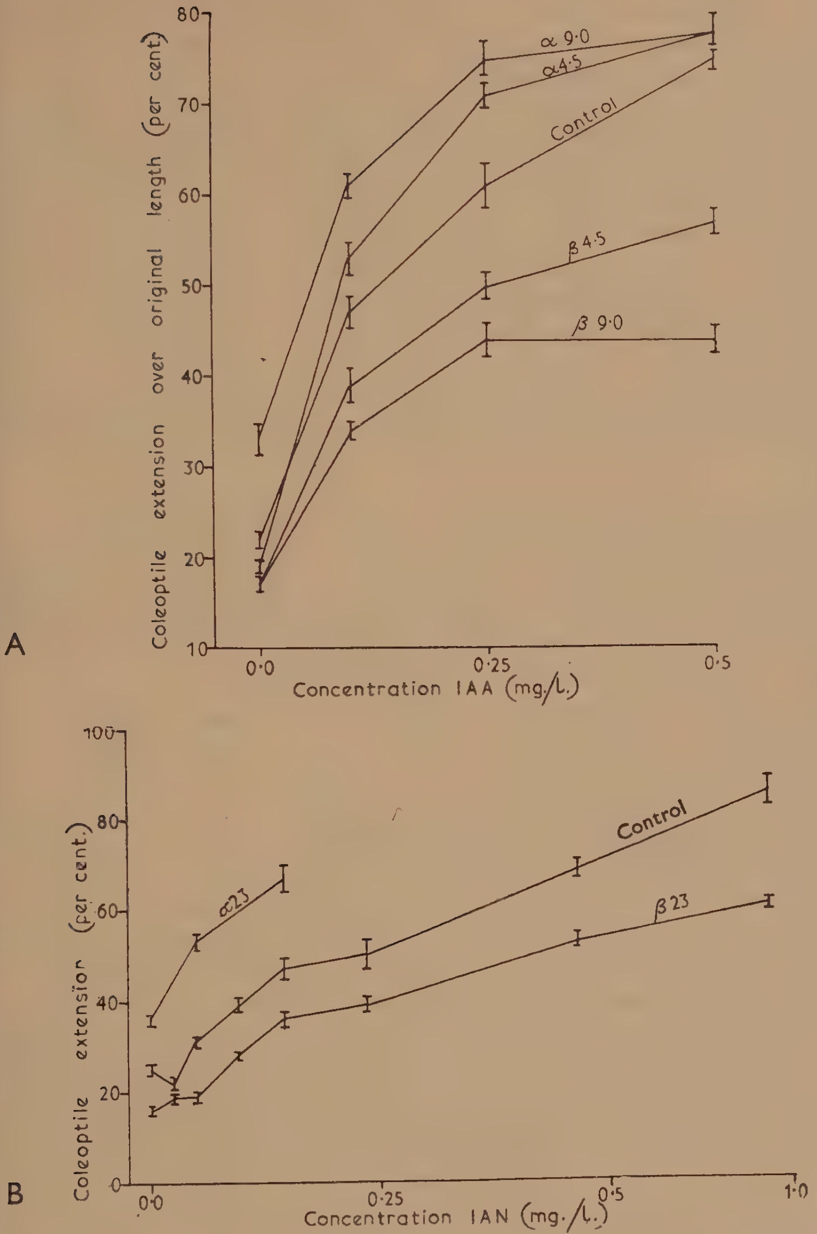

FIG. 4. Interaction in *Avena* coleoptile section extension of accelerator α and inhibitor β eluted from broad bean (4A) and pea (4B) shoot extract chromatograms with synthetic IAA (4A) and IAN (4B). The concentrations of α and β are normalities. The vertical lines are twice the standard errors.

obtained by elution from large-scale chromatograms. The IAA and IAN solutions used were made up from synthetic material. In addition to the procedures used in previous experiments, tests were made of the purity of the eluates by rechromatographing and bioassaying portions of them.

In an experiment in which broad-bean shoot was the source of α and β, two concentrations of each of these substances (4·5 N. and 9 N.) were tested with 0·0, 0·1, 0·25, and 0·5 mg./l. of IAA in the coleoptile section extension test. The results are graphed in Fig. 4A. Accelerator α was seen to promote and inhibitor β to inhibit the elongation induced in coleoptile sections by IAA. The promotion effect of α upon elongation would appear to hold only up to the optimum, which would mean that the maximum elongation would not be altered by the addition of α. This was proved in a test with mixtures of α with a wider range of IAA concentrations. Eluates of 23 N. α and 23 N. β, from a chromatogram of pea shoot extract, have interacted with IAN in the experiment, the results of which are shown in Fig. 4B. It will be noted that the effects of α and β upon the *Avena* coleoptile extension due to IAA or IAN are very similar.

The results of the measurements of *Avena* coleoptile section growth at a number of time intervals have been plotted as histograms of extension rates against time. The solutions of α and β used in these experiments were eluted from a potato tuber extract chromatogram. Coleoptile sections were treated with 0·1, 1·0, and 10·0 mg./l. of IAA, alone and in mixtures with 11·5 N. α and the extension rates are plotted against time in Fig. 5A. Although this concentration of α stimulated the growth of the control, it inhibited the growth of those sections treated with IAA. The extension rate-time histograms show that the stimulatory effect of α upon the control growth rate occurred mainly during the early stages of growth, i.e. up to about 10 hours. It was also at this point that the inhibiting effect of 11·5 N. α in mixtures with IAA became apparent. In an experiment that was done with 46 N. α, which had a marked inhibitory effect upon elongation in IAA solutions, there was little inhibition in the first 5 hours but between 5 and 10 hours the growth fell sharply, then flattened out. In Fig. 5B are the results of an experiment in which coleoptile sections were grown in 0·1, 1·0, and 10·0 mg./l. of IAA alone and in mixtures with 46 N. β. These results reveal the previously unexpected fact that inhibitor β promoted section growth in the control during the first 5 hours, after which it inhibited the growth rate. When β was mixed with IAA it was a growth inhibitor over the whole time period, but the greatest reduction in growth rate occurred between 5 and 10 hours from the start.

DISCUSSION

The results of the experiments with α and β have given a more complete picture of the properties of these substances with respect to elongation growth in coleoptiles and roots. These properties would be of little consequence if they occurred at concentrations of α and β that could not be considered as physiological. The empirical concentration scale that was adopted would give

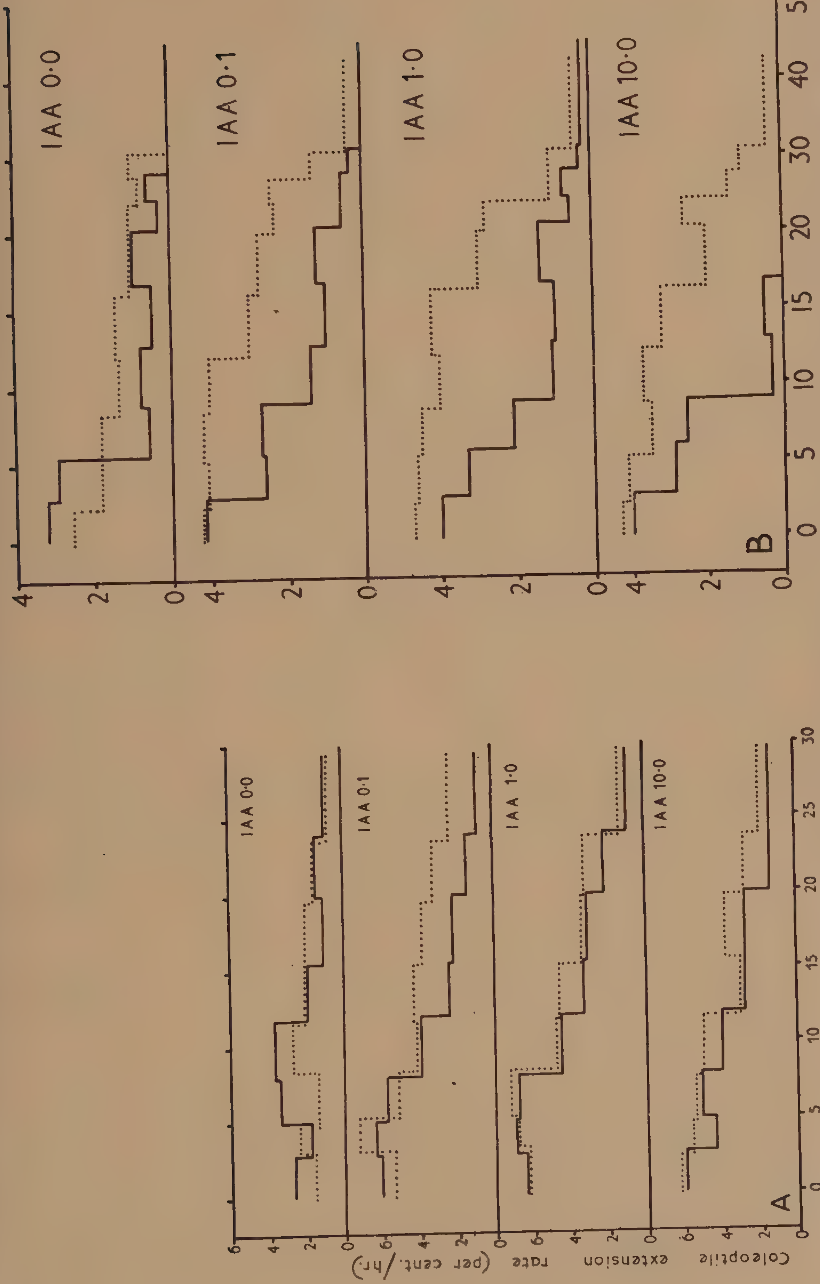

FIG. 5. The extension rate-time relationships for *Avena* coleoptile sections in 0·0, 0·1, 1·0, and 10·0 mg./l. of IAA alone (dotted lines) and in mixtures (full lines) with 11 N. accelerator α (5A) and 46 N. inhibitor β (5B). The α and β were eluted from a potato tuber extract chromatogram. Times are given in hours and the time scale in 5B is contracted after 20 hours.

approximately the concentration of the substances if evenly distributed in the plant water. Since the calculations of normality were based on the whole seedling and experiments in Part I (Kefford, 1955) showed that α, IAA, and β were concentrated in certain parts, the normality only gives an approximate estimate of the concentrations of the substances in those parts where they are most active.

Accelerator α was found to promote, to a relatively small extent, the extension growth of sections from roots and coleoptiles and at higher concentrations, which might still be physiological, it was inhibitory to both types of sections. The promotion of growth in roots, although always detected, was not as pronounced as that in coleoptiles. Both the promotion of growth in coleoptiles and roots by α have been confirmed by Lexander (1953), who used intact wheat roots as her root test material. The fact, seen in Fig. 3*a* and 3*c*, that α promoted root and coleoptile elongation at approximately the same concentration was of considerable interest, since with IAA there is a great difference between the concentrations that give promotion to the growth in roots and coleoptiles. Maximum elongation by IAA in root sections occurs at about 10^{-11} M. and in coleoptile sections at about 10^{-5} M. In mixtures with IAA and IAN, α was found to stimulate the elongation induced in coleoptiles by these substances even in concentrations at which α alone had little effect upon growth. The results presented in Fig. 5A show that higher α concentrations which stimulate coleoptile growth can inhibit IAA induced elongation. In its own effect upon coleoptile elongation, α appeared to change rapidly, with increasing concentration from a promotor to an inhibitor of elongation. Thus α could act as a 'fine adjustment' in growth control. Stowe and Thimann (1954) have suggested that α is indole-3-pyruvic acid, which is readily oxidized to IAA and may owe its growth activity to such a conversion. The root and coleoptile growth-concentration curves for α would be difficult to explain on the basis of a plain conversion to IAA, even if it could be shown that pea root sections were much less efficient in the indolepyruvic acid to IAA conversion than *Avena* coleoptile sections. On the chromatograms of plant extracts (see Part I of this series), α was found to spread over at least 5 cm. and there was the possibility that there were two substances present. Thus in some of the elongation experiments the area considered to hold α was divided into two parts and the eluates were tested separately, but the results gave no indication of the presence of two substances.

Inhibitor β inhibited the growth of root and coleoptile sections at all concentrations and it inhibited the IAA and IAN induced elongation of coleoptile sections. The eluates considered to contain IAA gave the growth *v.* concentration curves that would be expected for IAA, thus adding further evidence for the identity of the growth substance running on extract chromatograms at the same place as synthetic IAA. Figs. 2*b* and 3*b* are of interest since they are graphs of a root section test upon IAA from a root extract and a coleoptile section test upon IAA from a shoot extract, respectively. These figures present good evidence for the natural IAA in roots being inhibitory to their growth while the reverse holds for shoots. This point has been demonstrated elegantly by Lexander (1953).

The results given here will serve to emphasize the impossibility of treating the growth response by an *Avena* coleoptile as an estimate of the IAA content of an ether extract of plant material. It was at first thought that one could apply an enzyme-kinetic treatment of the type discussed by McRae and Bonner (see also Bennet-Clark and Kefford, 1954; Housley, Bentley, and Bickle, 1954; Bonner and Foster, 1955) to the interactions of IAA with α or β.

This is at present not possible. Data given above show that the procedure of bioassay by measurement of growth during, say, a 20-hour period provides a too incomplete assay. β, which we termed an inhibitor, by itself in absence of IAA has growth accelerating action.

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Studies on Fat Accumulation by Algae

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SUMMARY

The generally held view that fat accumulation is characteristic of certain classes of algae is based on inadequate evidence.

In this investigation the fatty acid, unsaponifiable lipoid, total cell nitrogen, and hydrolysable polysaccharide contents of the following algae have been determined at various stages during growth in pure culture: *Chlorella vulgaris*, *Scotiella* sp., *Euglena gracilis* var. *bacillaris*, *Tribonema aequale*, *Monodus subterraneus*, *Navicula pelliculosa*, *Porphyridium cruentum*, *Anabaena cylindrica*, and *Oscillatoria* sp.

Using the results obtained, the capacities of the algae to accumulate fat have been compared in the following ways: (1) on a percentage dry-weight basis; (2) by means of the differential growth factor relating the fat content of cultures to dry weight of algal material; (3) by consideration of the proportion which fats form of the total reserve material (taken as fat plus hydrolysable polysaccharide) as a function of total cell nitrogen content.

Species belonging to the same class have been found to resemble each other in the relative amounts of crude protein, fats, and hydrolysable polysaccharide which they contain. Marked differences in composition between species belonging to different classes have been found, but it is concluded that there are no fundamental differences in the physiological relations of fat accumulation in the algae belonging to the Chlorophyceae, Euglenineae, Xanthophyceae, and Bacillariophyceae. Representatives of the Rhodophyceae and Myxophyceae, although they may under certain circumstances have moderately high fat contents, appear to differ in that in them fat accumulation is not associated with low cell nitrogen contents as it is in the other algae examined.

No well-defined correlations between the unsaponifiable lipoid and either the fatty acid or total cell nitrogen contents of the algae have been found. *Euglena gracilis* var. *bacillaris* has been found to have a rather higher content of unsaponifiable lipoid than the other algae.

INTRODUCTION

THE accumulation of fats or oils has generally been regarded as a characteristic of certain classes of algae (see, for instance, Fritsch, 1935, pp. 5–9; Smith, 1951, pp. 16–17). However, few determinations of such substances in algae have been made and the evidence for the generalization is mainly derived from qualitative tests incapable of distinguishing between true fats and substances of quite different chemical nature such as are included in the analytical fraction 'unsaponifiable lipoid'. It is apparent, moreover, that the fat content of a given species can vary considerably according to its physiological condition (von Witsch, 1948; Spoehr and Milner, 1949; von Denffer, 1949; Aach, 1952; Fogg, 1953), so that it may be suspected that the accumulation of high proportions of fat is dependent on the environment in which a species is

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normally found rather than upon an intrinsic tendency of the organism to fat synthesis (Ketchum and Redfield, 1949; Myers, 1951). If intrinsic differences in this respect do exist they would appear to differ in nature from the well-substantiated differences in accessory photosynthetic pigments and carbohydrates which distinguish the algal classes, since, from the evidence at present available, there seem to be no important qualitative differences between the fats of species belonging to various classes (Fogg, 1953, p. 98), the apparent differences being only in the total quantity of fat accumulated. In the investigations described here and, in a preliminary way, in a previous paper (Fogg and Collyer, 1953) a number of species of algae has been examined in an attempt to determine whether or not such quantitative differences in fat accumulation between the algal classes do exist.

METHODS

General considerations. The variation in fat content which is found according to the conditions under which growth has taken place makes direct comparison of fat accumulation in different species impossible. Valid comparison can only be made between samples grown under similar conditions and representing equivalent stages of growth, but such samples cannot be obtained when, as nearly always happens, the algae concerned have different growth requirements. Standard culture conditions will not suffice for good growth of a sufficient variety of species for an investigation of this sort, a point which is illustrated by the results of an experiment in which three species were grown under identical conditions and which gave a most misleading idea of their relative abilities to accumulate fat (see Table IX). Hence it seems that comparisons of different species can only be indirect and should allow for variations in the physiological state of the material. In this investigation each species studied was grown in a medium suited to its particular needs, but culture conditions were otherwise kept as far as possible the same. Nitrogen deficiency appears to be a factor of preponderating importance in promoting fat accumulation in many micro-organisms (see p. 266) and accordingly nitrogen was supplied in the media at the lowest level consistent with obtaining a yield of algal material sufficient for analysis. Besides determinations of fatty acids and unsaponifiable lipoid, dry weight, total combined nitrogen content, and hydrolysable polysaccharide were determined for each sample so that different bases for consideration of the results should be available.

Organisms and culture methods. The algae studied, all of which were in unialgal bacteria-free culture, were as follows:

CHLOROPHYCEAE: *Chlorella vulgaris* Beij.; strain isolated by Hopkins and Wann (see Pearsall and Loose, 1937): *Scotiella* sp.; isolated from a wet rock face, Windermere (National grid ref. SD/388956), purified from bacteria by treatment with ultra-violet light: this hitherto undescribed species, which has some resemblance to an *Oocystis*, has tentatively been placed in the genus *Scotiella* by Dr. J. W. G. Lund, who will publish a description of it in due course.

EUGLENINEAE: *Euglena gracilis* Klebs var. *bacillaris* Pringsheim; Cambridge type culture collection No. 1224/7.

XANTHOPHYCEAE: *Monodus subterraneus* Petersen; strain isolated by Dr. R. A. Lewin;

Tribonema aequale Pascher; Cambridge type culture collection No. 880/1.

BACILLARIOPHYCEAE: *Navicula pelliculosa* (Bréb.) Hilse; strain isolated by Dr. J. C. Lewin (Lewin, 1953).

RHODOPHYCEAE: *Porphyridium cruentum* (Ag.) Naeg.; Cambridge type culture collection No. 1380/1.

MYXOPHYCEAE: *Oscillatoria* sp.; strain E2 of Allen (1952);

Anabaena cylindrica Lemm.; strain isolated by Fogg (1942).

The culture media used are specified in the tables of results. 'Hysil' Drechsel bottles fitted with sintered glass distributors and containing up to 500 ml. of medium were used as culture vessels. The cultures, after uniform inoculation with the algal suspension, were maintained at 25° C. in continuous light from a tungsten lamp giving an intensity of 6,500 metre-candles when measured with the test surface of a Holophane-Edgcumbe 'Autophotometer' in the position of the axis of one of the culture bottles and normal to the incident light. Aeration was at the rate of 10 litres per hour per culture with a mixture, containing (unless otherwise stated) 0·5 per cent. of carbon dioxide, obtained by combining air from a diaphragm pump and cylinder carbon dioxide, the amounts being regulated by calibrated flowmeters. Suitable precautions were taken to keep the cultures uncontaminated, results from contaminated cultures, which were detected by inoculation of samples into four different bacteriological media (as used by Fogg, 1951), being discarded. Eight cultures could be grown at a time and pairs of these were taken at intervals for analysis. The first samples were taken as soon as possible after the end of exponential growth and others at different times subsequently.

Analytical methods. The alga from a culture was collected by centrifugation and washed twice with distilled water. It was then resuspended uniformly in distilled water and aliquots of the suspension taken for the various analyses. *Oscillatoria* could not be dealt with in this manner, but, after washing, the whole sample was dried to constant weight and portions of this used for analysis.

The following determinations were made in duplicate upon each sample:

1. Dry weight: material was dried for 30 minutes at 95° C. and then to constant weight *in vacuo* at 65° C.

2. Fatty acids and unsaponifiable lipoid: the methods examined were those of Milner (1948) and Belin (1926). It was found difficult to obtain complete extraction of the fats of certain algae by Milner's method, and Belin's method of preliminary extraction with ethyl alcohol followed by saponification was generally used in this investigation. Extractions were carried out directly on fresh material centrifuged down from the suspensions. For complete recovery of fats from some species it was found necessary to saponify the residue as well

as the extract. The possibility of omitting the preliminary extraction and saponifying the material directly was examined. The results obtained in this way with *Chlorella* and *Anabaena* are distinctly low as compared with those obtained with preliminary extraction, but greater proportions of fat were obtained from *Monodus* after direct saponification. Direct saponification procedures are unsuitable for use with diatoms since the saponified extracts form a silica gel upon acidification, so that their further manipulation becomes difficult. In giving the results for each alga the method of extraction found to be most satisfactory is specified (see Tables I–VIII). Both the fatty acid and unsaponifiable fractions were dried to constant weight at 50° C. in an atmosphere of oxygen-free nitrogen.

3. Total combined nitrogen: a conventional micro-Kjeldahl method was used with a selenate mixture as catalyst in the digestion.

4. Total hydrolysable polysaccharide: estimated as glucose by a modified Hagedorn-Jensen method (Hulme and Narain, 1931) after hydrolysis with 3 per cent. sulphuric acid for 3 hours at 100° C. (Pearsall and Loose, 1937). No correction was made for free reducing sugars which might have been present initially.

RESULTS

The analytical results obtained are presented in Tables I–IX. Results for *Monodus* and for molybdenum-deficient *Anabaena*, in neither of which was hydrolysable polysaccharide determined, have already been given in full in the previous paper (Fogg and Collyer, 1953).

For most of the algae reasonable agreement was found between results obtained with duplicate cultures. Cultures of the filamentous forms, *Tribonema* and *Oscillatoria*, however, were far from uniform and in duplicate cultures the relative amounts of growth occurring in the bulk of the medium, on the sintered glass, or on the sides of the vessel above the level of the medium were sometimes widely different.

The compositions upon a total dry-weight basis of the different species at various stages of growth are given in Fig. 1. For this purpose protein has been taken as amount of nitrogen × 6·25 and, assuming that the fatty acids are chiefly unsaturated C_{16} to C_{20} acids (see Table VII, Fogg, 1953) present in the organism as triglycerides (see Kathen, 1949), fats have been taken as amount of fatty acids × 1·045.

The general tendency for the protein content of the algae to fall as the cultures age is clear. Except for *Monodus*, *Euglena*, *Porphyridium*, and *Anabaena*, nitrogen was the major factor limiting growth in these cultures, since the nitrogen supplied was completely recovered in the alga in the older cultures. At the time when these experiments were made a medium giving optimum growth of *Monodus* had not been devised and growth was limited by deficiency of some element other than nitrogen. *Euglena* requires organic growth factors (see Cramer and Myers, 1952) and it seems that, even in the medium containing $\frac{1}{10}$th of the normal amount of combined nitrogen, growth

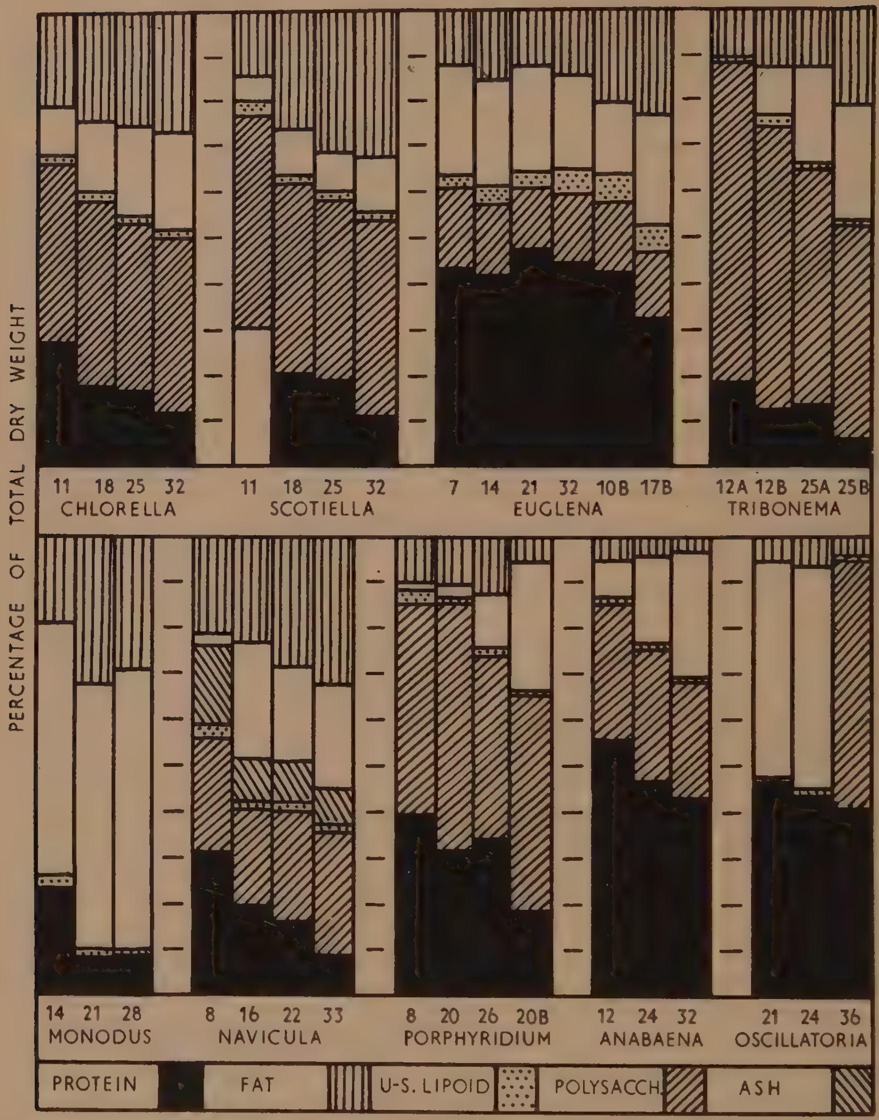

FIG. 1. Histograms showing the composition of various algae grown in culture. Amounts of protein, fat, unsaponifiable lipoid, hydrolysable polysaccharide, and ash are expressed as percentages of the total dry weight. The figure under each column denotes the age in days of the cultures from which the material was obtained. The sum of the various fractions in 36-day-old *Oscillatoria* amounts to 111·5 % (see p. 261 of text). For other details see the tables.

was limited by the amounts of these factors present rather than by nitrogen deficiency. In this species the level of protein remained high as the cultures aged, falling appreciably only in old cultures in the nitrogen-deficient medium. *Porphyridium* has complex and, as yet, incompletely defined growth requirements (Pringsheim and Pringsheim, 1949), and in our cultures its growth was limited by some other factor than supply of nitrogen. *Anabaena* is able to fix the free nitrogen of the atmosphere and for this species the fall in protein content with age was consequently slight.

It will be seen from the tables that in many cultures the amount of combined nitrogen recovered in the alga exceeded that originally supplied in the medium. The possible errors in the micro-Kjeldahl method mostly lead to underestimation rather than overestimation, and after consideration of our sampling and analytical techniques we conclude that increases in total combined nitrogen did actually occur in these cultures. Spoehr and Milner (1949) observed similar increases in combined nitrogen in cultures of *Chlorella pyrenoidosa* grown under conditions comparable to those used by us and tentatively attributed this to nitrogen fixation. However, it seems more probable that, both in our cultures and those of Spoehr and Milner, absorption from the air supplied of traces of combined nitrogen such as ammonia and oxides of nitrogen occurred rather than fixation of the free element. A growth of nitrogen-deficient alga will form an efficient absorbing system for traces of combined nitrogen, whereas thorough washing is necessary to remove small amounts of ammonia and oxides of nitrogen from air (Fogg, 1942). No precautions were taken in the present investigation to purify the air used, and, since aeration was vigorous and continuous, minute proportions of impurities in it would suffice to account for the observed increases in the combined nitrogen of the cultures.

A reciprocal relationship between the fat and protein contents in *Chlorella*, *Scotiella*, *Euglena*, *Tribonema*, *Monodus*, and *Navicula* is evident in Fig. 1. In *Porphyridium*, *Oscillatoria*, and *Anabaena*, on the other hand, the tendency was for the amounts of fat and protein to be directly proportional. In most species the proportion of unsaponifiable lipoid remained at about the same level or tended to fall as the cultures aged, showing no correlation with the proportion of fatty acids. In *Euglena*, however, the proportion of this fraction showed an increase with the physiological age of the cultures and reached a value substantially greater than that found for any other alga in this investigation. The proportion of hydrolysable polysaccharide did not change much with age of culture in any of the species examined. Only in *Tribonema* did this fraction tend to diminish as the cultures became physiologically older. The sum of the percentages of protein, fat, unsaponifiable lipoid, and hydrolysable polysaccharide in 36-day-old *Oscillatoria* cultures is 111·5. It seems likely that this is mainly due to an overestimation of hydrolysable polysaccharide. This fraction has been estimated as equivalents of glucose and the presence of materials yielding other reducing substances upon hydrolysis, e.g. pentosans, which have been found in appreciable quantity in certain blue-green algae (Hough, Jones, and Wadman, 1952), might lead to considerable error in its determination. In the other algae, however, a greater or lesser fraction of the dry weight was unaccounted for. Except for *Navicula*, in which the ash content was estimated, inorganic constituents must have made up an appreciable proportion of this fraction, but in *Chlorella*, *Scotiella*, *Tribonema*, *Porphyridium*, and *Anabaena* a large part of the unestimated material was probably cell-wall materials such as would not be hydrolysed under the conditions used by us or which do not yield reducing products on hydrolysis. It is possible that in *Porphyridium* sugar alcohols formed an appreciable proportion of the unestimated fraction. As far as our present inadequate knowledge of the chemical constituents of the Euglenineae and Bacillariophyceae goes, these explanations would not appear to suffice for the *Euglena* and *Navicula* results and it is clear that more detailed chemical investigation of these algae is desirable.

The relation of fat accumulation to growth in any one experiment may be simply expressed in terms of the differential growth factor, k, as defined in the expression

$$\log f = a + k \log w,$$

where f and w are respectively the amounts of fatty acids and the dry weight of algal material present in a culture and a is a constant. The value of this allometric method in biochemistry was pointed out by Needham (1932) and it has previously been applied in studies of growing cultures by Fogg (1949, 1952). It is not to be expected that k will remain constant for a given species grown under various environmental conditions. For example, although the numerical data given are inadequate for plotting in this way, it is evident from the results of Spoehr and Milner (1949) that for *Chlorella pyrenoidosa* grown with excess of nitrate k would be approximately unity, whereas under conditions of nitrogen deficiency it is considerably greater than unity (see Fig. 2). The advantage of using k is that it represents in a single figure the capacity of an organism to accumulate fat under a given set of circumstances. Ideally, the range of k should be determined for each species studied. However, this would be a matter of considerable labour and the best that can be done with the data available is to assess how far the k values obtained represent the performance of the species studied under optimum conditions for fat accumulation.

When the total amount of fatty acid per culture is plotted against dry weight of alga per culture on a logarithmic grid the points fall along straight lines for most species studied (Figs. 2 and 3), showing that k is nearly constant under the conditions of culture used. k and its standard deviation for each set of results have been calculated by the usual method of least squares for error in one variate, a method which, although only approximate when used with these data, gives sufficiently accurate results because the error in determination of dry weight is relatively small. The statistical methods and notation used are those described by Mather (1946).

In the analysis by this method, data for the same species from different experiments have been combined. Only for *Chlorella vulgaris* (Fig. 2) is there a marked heterogeneity amongst the combined data and attention has consequently been confined to the results for this species given in Table I. In calculating the line of best fit for *Tribonema* (Fig. 2) the results for one of the 12-day cultures, series B (Table IV), have been discarded on the grounds that the growth was abnormally low (see p. 259). Some results obtained by Aach (1952, fig. 13) for *Chlorella pyrenoidosa* have also been examined by logarithmic plotting for comparison (Fig. 2).

The algae examined fall clearly into two groups according to this method of analysis, those for which k is greater than 1, i.e. algae in which the proportion of fatty acids increased with age, and those for which k is less than 1, i.e. algae in which the proportion of fatty acids decreased with age. *Chlorella vulgaris*, *C. pyrenoidosa*, *Scotiella*, *Monodus*, *Tribonema*, and *Navicula* belong to the first group, *Euglena*, *Porphyridium*, *Oscillatoria*, and *Anabaena* to the second. The differences of k from unity are mostly statistically significant at the $P = 0{\cdot}05$ level. Differences between k values for sets of results from the two groups are statistically significant with the exception of that for *Tribonema* and *Porphyridium*, which barely reaches the significant level ($d = 1{\cdot}028 \pm 0{\cdot}469$, $t_{[7]} = 2{\cdot}2$, $P > 0{\cdot}05 < 0{\cdot}1$). These results are discussed further on p. 267.

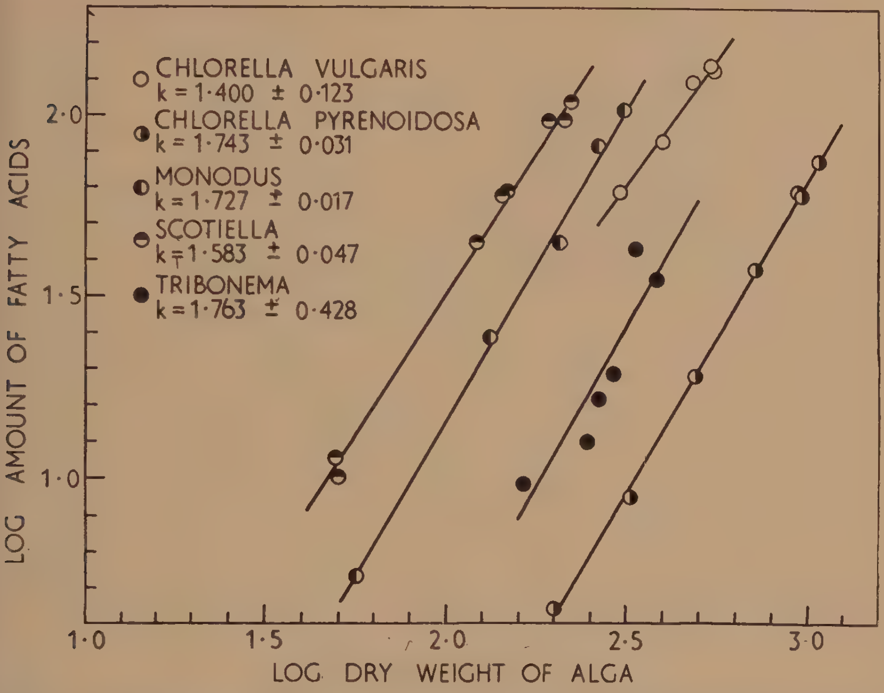

FIG. 2. Fat accumulation in cultures of *Chlorella vulgaris* (○), *C. pyrenoidosa* (◑), *Scotiella* sp. (◓), *Monodus subterraneus* (◐), and *Tribonema aequale* (●). Log$_{10}$ of amount of fatty acids per culture plotted against log$_{10}$ of dry weight of alga per culture. To avoid confusion, the points for some species have been displaced, those for *C. pyrenoidosa* 1·0 unit vertically downwards, those for *Scotiella* 0·2 unit horizontally to the left, and those for *Tribonema* 0·2 unit horizontally to the right. For explanation see text.

The positions of the regression lines in Figs. 2 and 3 give less useful information about the capacity of the algae to produce fatty acids than do the k

values. The position of a line depends on such variables as the size of the culture and the stage at which samples were taken and gives no information about the properties of the alga which cannot be conveyed more clearly otherwise.

It was suggested in our first paper (Fogg and Collyer, 1953) that it may be

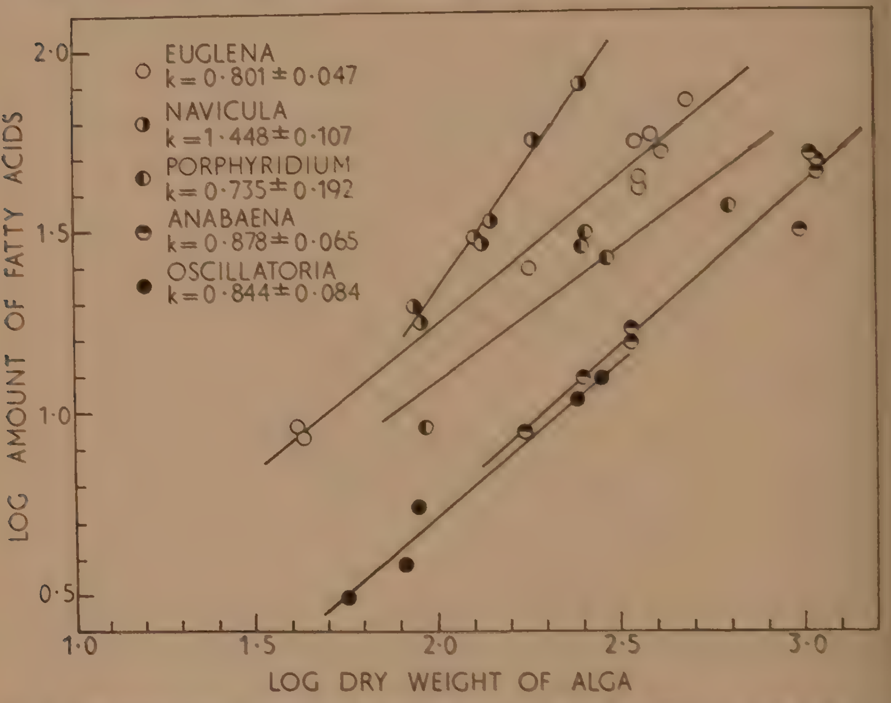

FIG. 3. Fat accumulation in cultures of *Euglena gracilis* var. *bacillaris* (○), *Navicula pelliculosa* (◑), *Porphyridium cruentum* (◐), *Anabaena cylindrica* (⊖), and *Oscillatoria* sp. (●). Log$_{10}$ of fatty acids per culture plotted against log$_{10}$ of dry weight of alga per culture. For explanation see text.

useful to consider the proportion which fatty acids form of the total reserve material as a function of the total amount of combined nitrogen in the cells. The data now available permit a further examination of this method. In our previous study the content of reserve material in an algal sample was taken as equal to total dry matter other than protein, a procedure which is open to the objection that it includes the ash fraction with the reserve products. In Fig. 4 amounts of fatty acids have been expressed as a percentage of the sum of the amounts of fatty acids and hydrolysable polysaccharide. While this sum does not necessarily include all the reserve products to be found in an alga, it has the advantage that it represents chemically more well-defined entities than residual dry matter. Insufficient determinations of the hydrolysable polysaccharide contents of *Monodus* and *Oscillatoria* were made for these species to be included in this consideration.

The results presented in Fig. 4 confirm the impressions gained from our previous study. Compared at corresponding levels of cell nitrogen the algae investigated differ markedly in the proportion of fatty acids in their reserve material. The two members of the Chlorophyceae and *Euglena* and *Navicula* produce high proportions of fatty acids, whereas *Tribonema*, *Porphyridium*, and *Anabaena* produce low proportions of these substances. However, in Fig. 4, as in Fig. 1 of our previous paper (Fogg and Collyer, 1953), a distinct tendency for the points for species belonging to the same class to lie together is evident. Birge and Juday (1922) have also noted that species belonging to the same class tend to be similar in general composition. Of perhaps fundamental significance is the differentiation of the classes into two groups according to whether the proportion of fatty acids rises or falls with increase in cell nitrogen level. This differentiation has been examined statistically by comparing the slopes (b) of the regression lines fitting the different sets of data. The following are the values for b with their standard deviations:

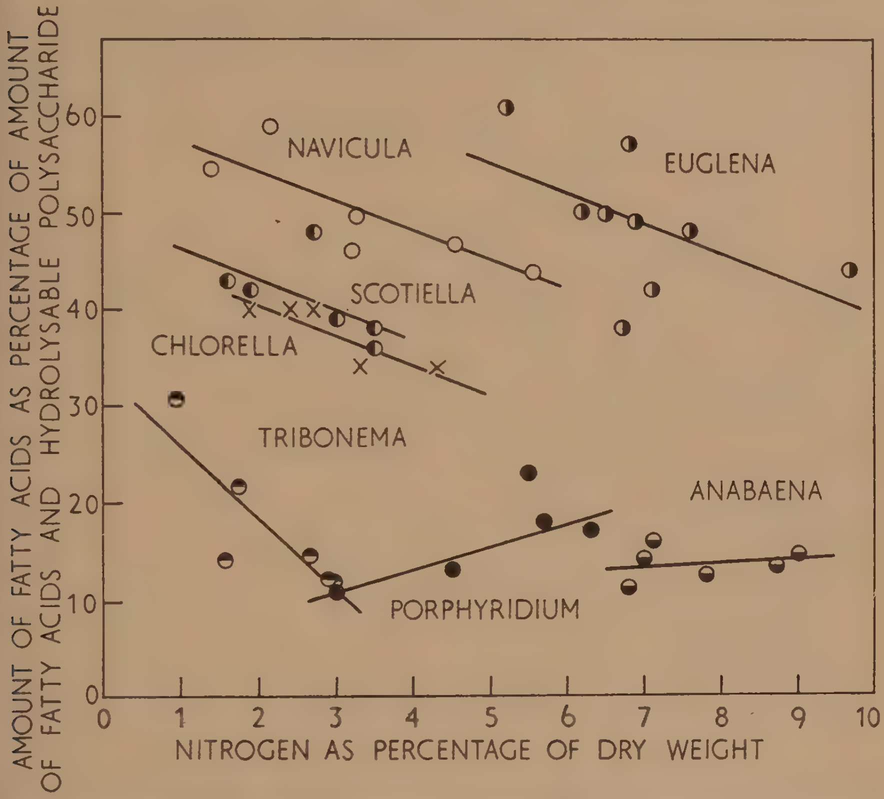

FIG. 4. Proportion of fatty acids in the reserve products of various algae as a function of cell nitrogen content. *Chlorella vulgaris* (×), *Scotiella* sp. (◐), *Euglena gracilis* var. *bacillaris* (◑), *Tribonema aequale* (⬒), *Navicula pelliculosa* (○), *Porphyridium cruentum* (●), and *Anabaena cylindrica* (◒).

Chlorella	−3·10±1·01	*Navicula*	−3·04±1·05
Scotiella	−3·10±2·17	*Porphyridium*	+2·65±1·42
Euglena	−3·14±1·90	*Anabaena*	+0·38±0·87
Tribonema	−7·53±2·52		

While the values for b for *Chlorella* ($t_{[3]} = 3{\cdot}07$, $P \sim 0{\cdot}05$), *Tribonema* ($t_{[4]} = 3{\cdot}0$, $P < 0{\cdot}05$), and *Navicula* ($t_{[4]} = 2{\cdot}9$, $P < 0{\cdot}05$) are statistically different from zero, the others are not. However, differences in b for certain pairs of species are statistically significant, e.g. those for *Euglena* and *Porphyridium* ($d = 5{\cdot}79 \pm 2{\cdot}37$, $t_{[10]} = 2{\cdot}44$, $P < 0{\cdot}05$) and for *Chlorella* and *Anabaena* ($d = 3{\cdot}48 \pm 1{\cdot}34$, $t_{[7]} = 2{\cdot}6$, $P < 0{\cdot}05$), and there thus appears to be a real difference between the two groups of results.

DISCUSSION

The interpretation of these results requires in the first place further consideration of the circumstances leading to the accumulation of fats by a micro-organism. Studies with a variety of higher plants and animals and fungi point to nitrogen deficiency as a major factor concerned (Kleinzeller, 1948) and this has been confirmed for *Chlorella pyrenoidosa* by von Witsch (1948), Spoehr and Milner (1949), Ketchum and Redfield (1949), and Aach (1952). Although the fat content of this alga can be varied experimentally by changes in any one of several factors, the primary limitation is imposed by the concentration of available nitrogen in the medium and only when this falls below a certain level can fat accumulation be considerable. Less extensive data point to fat accumulation in diatoms being determined in a similar manner (Beijerinck, 1904; von Denffer, 1949). It seems, however, that neither the concentration nor the nature of the nitrogen supply affect fat accumulation directly. Heide (1939) found that in a continuously renewed medium containing a low concentration of asparagine *Endomyces vernalis* did not accumulate fat, whereas it did under otherwise similar conditions when the nitrogen supply was allowed to become exhausted. The same worker found that equimolar concentrations of various inorganic and organic nitrogen compounds had similar effects on fat accumulation. Fat accumulation by *Chlorella pyrenoidosa* was found by Spoehr and Milner (1949) to be favoured by the supply of ammonium rather than of nitrate nitrogen, but higher concentrations of these two substances had similar inhibitory effects on the process. It seems therefore that the effects of nitrogen supply upon the synthesis of fats are exerted via some intermediate or product of nitrogen metabolism rather than directly by the substance supplied.

A factor which may be of great importance for fat accumulation, namely water deficiency, has received little attention in recent studies with algae and was not, in fact, taken into consideration by us until late in this investigation. Lüders (1862) observed that diatoms allowed to dry out accumulated conspicuous amounts of fat (it appears, however, that the only criterion used for its identification was the refractive nature of the drops) and that these disappeared when water was supplied again. Russell-Wells (1932) found the proportion of fat in different species of littoral marine algae to be correlated with the degree of exposure to which they were subjected. Halden (1934) demonstrated a striking increase in the amounts both of fats and sterols in yeast upon partial drying. These observations do not supply conclusive proof that deficiency of water itself promotes fat accumulation, since under the conditions to which the organisms were exposed deficiency of nutrients, including nitrogen, would inevitably be associated with it. However, the finding that high concentrations of salts bring about an increase in the fat content of *Endomyces vernalis* rather by their osmotic effect than by any specific action (Heide, 1939) suggests that a decreasing availability of water promotes fat accumulation independently of effects due to nitrogen deficiency. This idea is supported by an observation made in the course of the present investigation that *Oscillatoria*, in which nitrogen deficiency does not apparently induce fat accumulation, when grown on agar contained greatly increased amounts both of fatty acids and of unsaponifiable lipoids, viz. 11·7 and 4·1 per cent. of the dry weight respectively, as compared with the maximum values observed in liquid cultures with an otherwise similar medium. This increased production of fats as water becomes less available is teleologically understandable and would also be expected on the grounds that a decrease in the chemical potential of water dipoles within the protoplasm would favour an increase in the formation of non-polar groups at the expense of that of hydrophilic groups. It is to be suspected that the very high fat contents found in *Chlorella* by Spoehr and Milner (1949) result in part from the effect of water deficiency, since the harvested and still living material was dried at room temperature *in vacuo* for periods of up to 72 hours, a treatment similar to that which Halden (1934) found to be effective in producing high proportions of fats and sterols in yeast. However, it does not seem that this should invalidate their general conclusions regarding the effect of nitrogen on fat accumulation.

Other factors the effects of which have been investigated do not appear to exert effects of the same magnitude as those of nitrogen or water deficiency. High light intensities and low concentrations of oxygen favour accumulation in *Chlorella pyrenoidosa* provided that the alga is nitrogen deficient (Spoehr and Milner, 1949). Other factors, e.g. phosphate concentration, temperature, and carbon dioxide supply, are without perceptible effect (von Witsch, 1948; Spoehr and Milner, 1949; Ketchum and Redfield, 1949). Fat accumulation in *Nitzschia palea* occurs in the presence of an auto-toxin which blocks cell division without correspondingly affecting assimilation (von Denffer, 1948, 1949). It appears that this auto-toxin induces accumulation of fat irrespective of whether the cells are nitrogen deficient or not, but it would be desirable to have analytical data to confirm this point.

The progressive increase in the proportions of fat in the cells of *Chlorella*, *Scotiella*, *Tribonema*, and *Navicula* observed in our experiments can be related with reasonable confidence to the exhaustion of the nitrogen supplied in the medium. Deviations from a regular relationship such as are particularly noticeable for *Tribonema* (Fig. 2) are perhaps attributable to the effect of water deficiency in inducing fat accumulation in the variable amount of alga which collected on the sides of the culture vessels above the level of the medium. Interpretation of the data for the four species which in our cultures did not show an increase in proportion of fat with time is less straightforward. Exhaustion of the nitrogen supplied occurred in only one of the five *Oscillatoria* cultures analysed. However, the data for this culture seem to be in good agreement with those from the others (Fig. 3), and it seems that, at least under the experimental conditions used, nitrogen deficiency does not induce accumulation of fat in this species. *Anabaena* does not become nitrogen deficient unless nitrogen fixation is inhibited. This was achieved in the two molybdenum-deficient cultures, the results for which lie on the line of best fit to the other points (Fig. 3), but in view of the differential effects of molybdenum deficiency on the nitrogen fractions of *Anabaena* (Wolfe, 1954) the interpretation of this is uncertain. The nitrogen supplied was not completely utilized in the *Euglena* and *Porphyridium* cultures and it remains for further experimentation to show whether, if nitrogen deficiency can be achieved in cultures of these algae without undue reduction of growth, greater accumulation of fat than that observed will take place. It seems likely that, as with *Tribonema*, the irregularity of the relationship between amount of fatty acids and dry weight shown by this group of species (Fig. 3) was due to the effects of local conditions of water deficiency in the cultures.

Since the concentration of the combined nitrogen supplied is evidently not itself directly determining for fat accumulation, the process may next be considered as a function of the concentration of nitrogenous substances within the cells. It may be noted that the *Monodus* results provide justification for this method of treatment, since, although the nitrogen supplied in the medium was not exhausted, the relationship of its fatty acid and cell nitrogen contents was found to be similar to that in *Tribonema*, which was grown in medium which did become exhausted of nitrogen. The simple explanation of the effect of nitrogen deficiency in inducing fat accumulation, originally put forward by Beijerinck (1904), is that such deficiency prevents the synthesis of protoplasm without affecting assimilation to a similar extent, so that the products of photosynthesis which would otherwise be used in protein and protoplasmic synthesis now appear as non-nitrogenous reserve products such as fat. If this were the whole story it would be expected that deficiency of other nutrients not directly concerned in photosynthesis would also cause the accumulation of fat. However, deficiencies of phosphate, magnesium, and potassium have been found not to have this effect (Spoehr and Milner, 1949) and the effect of nitrogen deficiency therefore appears to be a specific one. Furthermore, if the accumulation of fat resulted simply from a diversion of the products of photosynthesis into the synthesis of reserve material, the proportion which fats form of the total reserve materials of the cell would not necessarily alter on nitrogen starvation. Plotting fats as a percentage of the total reserve material as in Fig. 4, one might expect the points to fall along a horizontal line. This is evidently not so. In certain species a marked shift in favour of fat synthesis is associated with a lowered cell nitrogen content (Fig. 4). The mechanism of this effect is unknown at present. It may be that nitrogen deficiency brings about a change in the proportions of enzyme systems in favour of those concerned in fat synthesis (Fogg and Collyer, 1953; Fogg, 1953), an hypothesis which we are now attempting to verify experimentally; it may be that the proportions of the different systems remain the same but that those concerned in carbohydrate synthesis become saturated before those involved in fat synthesis; or some other mechanism may operate.

However this may be, the algae studied in this investigation appear to differ considerably among themselves in the effect which a lowered cell nitrogen content has upon their relative capacities to synthesize fat and reserve carbohydrate. Again, it is a matter of difficulty to separate intrinsic differences from those depending on the different environmental conditions under which the algae had to be grown. The results of Aach (1952, fig. 14) for *Chlorella pyrenoidosa*, grown under different conditions of light and nitrogen supply but without the complicating factor of varying availability of water, when plotted in the manner of Fig. 4 give a parabolic curve with a minimum fat production at about 6·5 per cent. of nitrogen in the cells and rising fat production at both higher and lower nitrogen contents. The minimum proportion of fat attained perhaps, though not necessarily, corresponds with the *élément constant* of fat which is not reduced on prolonged starvation of the organism (Belin, 1926). For *Chlorella*, *Scotiella*, *Tribonema*, *Monodus* (see fig. 1, Fogg and Collyer, 1953), and *Navicula* we have perhaps obtained the left-hand limb of such a curve. The results for *Euglena* show considerable scatter, but possibly they too fall on the left-hand limb of a parabola, in which case this species does not differ in the physiological relations of fat accumulation from the algae just mentioned. The curves for these algae evidently occupy different positions so that the minimum proportion of fat and the cell nitrogen content at which it is produced differ from species to species. The question that has now to be considered is whether the lines fitted to the points for *Anabaena*, *Oscillatoria* (see fig. 1, Fogg and Collyer, 1953), and *Porphyridium* represent the right-hand limb of parabolas similar to those for the other species and, if so, whether the left-hand limb would have been obtained if data had been obtained for more nitrogen-deficient material, or whether the curves for these three species are of a different form. Considering that nitrogen deficiency was obtained in the *Oscillatoria* cultures it seems likely that the latter alternative is the correct one and that even if lower cell nitrogen contents could have been achieved there would have been no increase in the relative proportion of fat produced. The relationships for *Anabaena* and *Porphyridium* appear to be of the same type as that for *Oscillatoria* and even at a nitrogen content as low as 3 per cent. on a dry-weight basis *Porphyridium* showed no signs of accumulating an increased proportion of fat. More observations with the same species under a wider range of conditions and with other representatives of the same classes are required, but on the present evidence it would appear that members of the Rhodophyceae and Myxophyceae differ from those of other classes examined in showing no increased tendency to accumulate fat as the cell nitrogen content falls. This is not to say that red and blue-green algae are necessarily incapable of producing increased proportions of fat in response to changes in other factors, e.g. availability of water, the effects of which are independent of those of nitrogen deficiency.

The unsaponifiable lipoid fraction includes a variety of different components which almost certainly vary independently when the conditions under which the alga is grown are changed and it does not seem profitable to discuss the variations in the amount of this fraction in detail. There are no well-defined correlations between the amounts of unsaponifiable lipoid and either total nitrogen or fat contents in the algae studied, nor would these be expected. The single observation on *Oscillatoria* indicates that under conditions of water deficiency the proportion of unsaponifiable lipoid increases as well as that of fat (see p. 267). Halden (1934) observed a parallel increase in sterols and fats with increasing water deficiency in yeast and this would be expected to be a general effect of decreasing the chemical potential of water within the cells (see p. 267). *Euglena* shows a tendency to contain rather greater, and the two blue-green algae tend to contain rather smaller, proportions of unsaponifiable lipoid than do the other algae studied. While the differences observed in content of unsaponifiable lipoid in this investigation are not very great, the proportion of this fraction in *Botryococcus* has been found to be markedly higher (23 per cent. on a dry-weight basis as compared with 7 per cent. of fatty acids) than any recorded by us (see Belcher and Fogg, 1954), a finding which lends support to our contention that the usual qualitative tests may give misleading evidence as to an alga's capacity to accumulate fat.

In this investigation no important differences in the physiological relations of fat accumulation have been found between representatives of the Chlorophyceae, Euglenineae, Xanthophyceae, and Bacillariophyceae. This runs counter to the generally accepted view that fat accumulation is especially characteristic of the Xanthophyceae and Bacillariophyceae but is in agreement with numerous observations of conspicuous fat-like droplets within the cells that have, in fact, been recorded for species belonging to all four of these classes (see Fritsch, 1935). The representatives of the Rhodophyceae and Myxophyceae that have been examined, although they may under some circumstances have a moderately high fat content, appear to differ in an important respect from the other algae in that in them fat accumulation is not associated with low cell nitrogen contents. This conclusion is supported by observations that fat droplets do not occur in any considerable quantity in the cells of red and blue-green algae (Fritsch, 1945, pp. 411 and 786) and by the low fat contents that have previously been recorded for red (Macpherson and Young, 1949) and blue-green algae (Birge and Juday, 1922). It appears, then, that the Rhodophyceae and Myxophyceae possess in this a biochemical characteristic which differentiates them from certain of the other algal classes.

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TABLE I

Analyses of Chlorella vulgaris

Medium: C2 of Spoehr and Milner (1949) + ferric citrate (Rodhe, 1948); 12·6 mg. N supplied per culture; aeration with 2·5% CO_2 in air; fat extraction by the unmodified Belin method; amounts in mg. per 400-ml. culture.

Age of culture (days)	11	18	18	25	32
Dry weight	302	484	401	552	536
Fatty acids	61·3	125·4	85·4	135·8	138·8
Unsaponifiable lipoid	6·6	10·2	8·8	9·9	9·6
Total nitrogen	13·1	11·7	13·2	14·7	10·1
Hydrolysable polysaccharide	119·3	189·2	168·4	201·5	205·3

TABLE II

Analyses of Scotiella sp.

Medium: No. 10 of Chu (1942) at twice normal concentration; 5·5 mg. N supplied per culture; aeration with 2·5% CO_2 in air; fat extraction by the unmodified Belin method; amounts in mg. per 400-ml. culture.

Age of culture (days)	11	11	18	18	25	25	32	32
Dry weight	77	79	189	222	302	227	331	348
Fatty acids	11·5	10·0	44·8	59·7	98·5	61·3	98·3	110·7
Unsaponifiable lipoid	2·6	2·7	3·6	4·4	5·7	4·1	5·3	4·5
Total nitrogen	—	—	6·6	6·7	8·2	7·9	6·3	5·6
Hydrolysable polysaccharide	34·9	38·2	79·9	93·0	109·0	99·4	138·4	148·6

TABLE III

Analyses of Euglena gracilis var. bacillaris

Media: Series A, sodium acetate hydrated 2 g., Lemco beef extract 2 g., Difco tryptone 2 g., Pyrex distilled water 1 litre; 195 mg. N supplied per culture; aeration with 2·5% CO_2 in air. Series B, as for A but with Lemco beef extract 0·2 g., Difco tryptone 0·2 g.; aeration with 0·5% CO_2 in air. The two series were grown on different occasions and from different amounts of inoculum; fat extraction by unmodified Belin method; amounts in mg. per 400-ml. culture.

	Series A							Series B	
Age of culture (days)	7	7	14	14	21	31	32	10	17
Dry weight	362	365	352	386	418	178	495	42·8	40·8
Fatty acids	40·5	43·8	54·6	57·1	51·4	24·4	70·8	8·5	9·2
Unsaponifiable lipoid	10·5	10·2	15·5	14·3	14·2	9·8	23·8	2·7	2·2
Total nitrogen	24·1	26·0	24·4	25·2	31·7	17·3	30·8	2·9	2·1
Hydrolysable polysaccharide	65·5	59·9	57·0	57·9	56·0	31·5	69·8	6·3	6·0

TABLE IV

Analyses of Tribonema aequale

Media: Series A, solution No. 10 (Chu, 1942) at twice normal conc., 5·5 mg. N supplied per culture. Series B, as A but with half the amount of calcium nitrate replaced by an equivalent of calcium chloride; both series grown under otherwise identical conditions; amounts of fat represent the total of that obtained by saponifying both alcohol extract and residue; amounts in mg. per 400-ml. culture.

	Series A				Series B		
Age of culture (days)	12	12	24	25	12	12	25
Dry weight	167	102	239	155	180	69	209
Fatty acids	16·7	9·7	35·4	12·7	19·4	11·1	43·1
Unsaponifiable lipoid	2·2	2·8	2·6	3·3	3·1	3·5	3·3
Total nitrogen	4·8	3·0	4·2	4·1	2·8	2·3	2·0
Hydrolysable polysaccharide	119·2	70·7	128·3	75·2	118·3	36·4	96·8

TABLE V

Analyses of Navicula pelliculosa

Medium: No. 10 (Chu, 1942) + 2% (V/V) soil extract (this addition was evidently unnecessary, see Lewin, 1953); 3·2 mg. N supplied per culture; fat extraction by unmodified Belin method; amounts in mg. per 400-ml. culture.

Age of culture (days)	8	8	16	16	22	22	33
Dry weight	88	92	141	135	186	128	253
Ash	13·5	17·0	14·0	10·5	14·5	14·5	20·0
Fatty acids	19·5	17·2	33·2	28·8	56·3	29·8	79·7
Unsaponifiable lipoid	3·0	3·0	2·7	3·0	3·7	3·3	4·2
Total nitrogen	4·0	5·1	—	4·4	4·0	4·1	3·6
Hydrolysable polysaccharide	22·4	22·0	27·9	29·5	39·3	35·1	66·3

TABLE VI

Analyses of Porphyridium cruentum

Medium: Seawater—Beijerinck solution + 0·2% Difco tryptone (Pringsheim and Pringsheim, 1949), 175 mg. N supplied per culture; aeration with 2·5% CO_2 in air. Series A and B were grown on different occasions from different amounts of inoculum; fat extraction by unmodified Belin method; amounts in mg. per 400-ml. culture.

	Series A				Series B
Age of culture (days)	8	20	20	26	20
Dry weight	94	254	297	256	635
Fatty acids	9·1	28·4	26·1	31·2	36·6
Unsaponifiable lipoid	3·0	5·1	2·7	4·4	5·7
Total nitrogen	5·9	14·4	13·4	14·1	18·7
Hydrolysable polysaccharide	43·1	127·5	169·8	102·1	295·3

TABLE VII

Analyses of Anabaena cylindrica

Medium: Nitrogen-free (Fogg, 1949); fat extraction by unmodified Belin method; the results given may be slightly low, as saponified residue from the extraction gave an emulsion with petroleum ether which could not be analysed further but which evidently contained a small proportion of fat; amounts in mg. per 500-ml. culture.

Age of culture (days)	11	11	23	24	30	33
Dry weight	252	341	1,050	1,091	1,095	969
Fatty acids	12·3	16·6	49·8	48·2	44·2	30·6
Unsaponifiable lipoid	2·4	5·4	16·8	14·5	9·0	7·8
Total nitrogen	21·8	30·6	81·5	78·0	76·1	66·2
Hydrolysable polysaccharide	79·6	96·5	349·7	253·1	269·4	241·3

TABLE VIII

Analyses of Oscillatoria sp.

Medium: No. 5 (Fogg and Collyer, 1953), 18 mg. N supplied per culture; aeration with 2·5 % CO_2 in air; fat extraction as for *Tribonema* (Table IV); amounts given in mg. per 400-ml. culture.

Age of culture (days)	21	24	36	36
Dry weight	56·8	88·8	279·5	243·1
Fatty acids	3·1	5·5	12·3	10·7
Unsaponifiable lipoid	0·3	1·3	2·0	2·9
Total nitrogen	4·2	6·2	20·6	13·7
Hydrolysable polysaccharide	—	—	178·3	161·2

TABLE IX

Analyses of Chlorella vulgaris, Monodus subterraneus, and Oscillatoria sp. grown under identical conditions

Medium: As for *Anabaena* (Fogg, 1949) + 0·2 g./litre KNO_3, 0·2 g./litre Na_2CO_3 and iron as ferric citrate (Rodhe, 1948); 6·9 mg. N supplied per culture; aeration with 2·5 % CO_2 in air; fat extractions: *Chlorella*, unmodified Belin method; *Monodus*, by direct saponification of dried material; *Oscillatoria*, as for *Tribonema* (Table IV); amounts given in mg. per 400-ml. culture; the results for *Oscillatoria* are means from 3 cultures which were combined for analysis.

	Chlorella		*Monodus*	*Oscillatoria*
Age of culture (days)	18	18	19	19
Dry weight	313	406	56	81
Fatty acids	93·3	149·4	5·4	3·8
Unsaponifiable lipoid	7·5	8·5	0·8	0·5
Total nitrogen	9·6	11·9	4·9	5·4
Hydrolysable polysaccharide	90·1	99·1	22·9	24·1

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Note added in proof

The organism designated as *Oscillatoria* sp. in this paper has been identified as *Microcoleus vaginatus* Gom. by Dr. F. Drouet, an identification confirmed by Dr. P. Bourrelly.

The Influence of the Cell Membrane on some Toxicity Effects of Growth Substances in *Nitella*

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SUMMARY

When cells of *Nitella* are placed in a solution of some plant growth substances there is a profound increase in the membrane resistance as measured by means of an internal silver/silver chloride micro-electrode. This impedance effect is accompanied by some marked visible changes within the cell cytoplasm, these changes being dependent upon the concentration of undissociated growth substance in the vicinity of the membrane, and upon the pH of the external environment.

A detailed study is made of these visible changes and, taking into account the negatively charged membrane, one possible interpretation of the results is that only undissociated molecules of the growth substance may enter the cytoplasm of *Nitella* from the environment, the diffusion obeying a simple Fick Law relationship.

The substances are used in sufficient quantity to kill the *Nitella* cell, death following the visible effects, and they appear to be unique in that their toxic effect produces a system of high membrane impedance.

INTRODUCTION

THE small volume of the cytoplasm associated with most living plant cells sets a limit on the use of normal analytical methods in finding its properties; it is extremely difficult, therefore, to investigate the equilibrium which must exist between cytoplasm and environment. However, all solutes passing between these two phases must pass through the cell membrane, the properties of which, being somewhat more amenable to measurement, may cast some light on the mechanism of the establishment and maintenance of equilibrium.

In a previous paper (Bennett and Rideal, 1954) a technique has been described whereby the electrical resistance and capacity of the membrane of *Nitella* can be measured. The effect of some growth substances was examined, and it was found that they caused a profound increase in membrane resistance, of the order of 350 per cent. The substances used included the sodium salts of indole-3-acetic acid, 2:4-dichlorophenoxyacetic acid, and 2-methyl-4-chlorophenoxyacetic acid; in some cases the presence or absence of Ca^{++} ions had marked effects on the resistance increases measured. The pH of the solution was found to be an important factor in determining the concentration

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of growth substance necessary to produce a resistance increase, and it was shown that the measured effects were due to the presence of the substance in its undissociated molecular form. However, the usual relationship between the concentration of molecules necessary to produce a resistance effect, the pK value of the substance, and the pH of the solution did not hold.

Peters (1931) made what he describes as 'the first measurement of the strength of ionization at an interface'. One of his conclusions was that the pH at a charged interface is different from that in the adjacent bulk phase, and Danielli (1937) has pointed out that the differences between bulk and surface concentration of hydrogen ions may be of the order of several pH units, and hence might be of great importance in biological processes. The phenomenon has been reviewed by Davies and Danielli (1951), who have studied some surface reactions involving a small diffusible molecule.

Under the conditions of the experiments on *Nitella*, recorded in the previous paper, the total ionic concentration always remained low, *c.* 0·0002N, and it appeared that the pH at the cell membrane *surface* was 1·11 units lower than that of the bulk external solution, so that the concentration of undissociated material in the vicinity of the membrane was greater than in the bulk solution.

The effect of pH on the biological activity of certain weak acids and bases has been reviewed in great detail by Simon and Blackman (1949) and Simon and Beevers (1952). They have studied the effects of aqueous solutions of various substances upon cell metabolism, and have shown that there is a general relationship between activity and the concentration of undissociated molecules of weak acid or base in the external solution. In all cases the test organism was examined under conditions of (dynamic) equilibrium and, in view of the great complexity of the system, clearly it would be desirable to consider the means by which this equilibrium is eventually reached.

When a *Nitella* cell was placed in a solution of growth substance certain visible changes took place within the cell. These changes appeared to be related to, and in fact led up to the profound membrane resistance increase and ultimate death of the cell. A detailed study was made of the visible changes produced by sodium 2-methyl-4-chlorophenoxyacetate (MCPA) and indole-3-acetic acid (IAA) in an attempt to ascertain whether the changes were in any way related to the penetration of these substances into the cytoplasm of the living *Nitella* cell.

EXPERIMENTAL METHODS

The *Nitella* used in this investigation was supplied by the Type-Culture Collection of Algae and Protozoa, Cambridge (N. sp. No. 776/1). It was cultured in glass vessels containing sterilized garden soil to a depth of 1 cm., a little calcium carbonate, and filled with tap-water. The vessels were illuminated by a 'warm-white' fluorescent tube 12 hours daily at laboratory temperature.

When a cell was placed in a solution of growth substance containing an appreciable concentration of the undissociated material (*c.* 50 mg./l., or 50 p.p.m.) whether an electrode had been introduced or not, two clearly visible effects could be noted: (*a*) after a time, t_1 minutes, cytoplasmic streaming stopped; (*b*) after a further time, t_2 minutes, a 'flood' of material suspended in the cytoplasm (chloroplasts and other particles) could be seen streaming rapidly towards either end of the cell, and this process was accompanied by a sharp rise in the measured membrane resistance. At the ends of the cell amorphous masses were seen to collect, and these darkened in colour a minute or two after their formation.

The measured and observed effects of different concentrations of IAA at pH_{bulk} 3·9 are shown in Fig. 1.

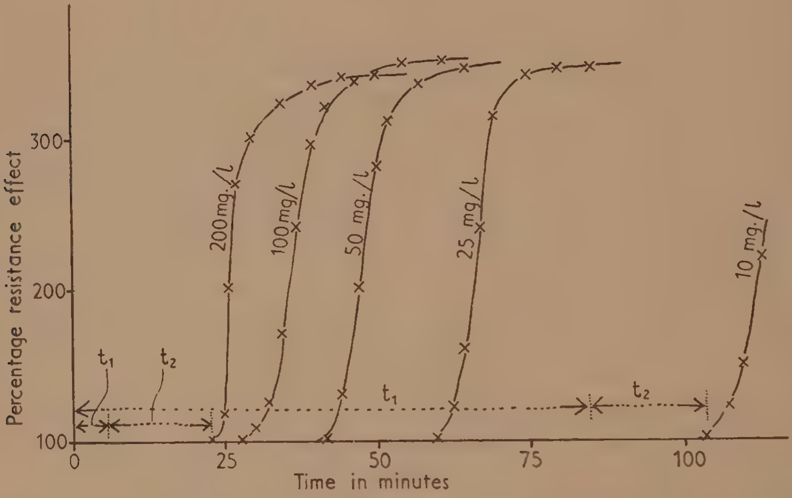


FIG. 1. Measured and observed effects of different concentrations 10 to 200 mg. l. of IAA at pH_{bulk} 3·9. t_1 is the time to stop cytoplasmic streaming, t_2 is the time to 'flood'. For other values of t_1 see Table II.

If a cell was removed from the solution of growth substance any time before the 'flood' was observed, and replaced in its culture medium, cytoplasmic streaming recommenced and returned to its original rate. After the 'flood' there was no evidence to suggest that the cell was still alive.

A series of experiments was carried out to investigate the effect of changes in concentration of growth substance and pH upon the times t_1 and t_2. The pH of the external solution was measured throughout each separate experiment by a Cambridge pH meter, the pH of each solution being reached only by the addition of a trace of HCl or NaOH, the total ionic concentration thereby remaining well below 0·001N.

The growth substances used were 98 per cent. pure and their melting points checked against known values. The samples were kindly supplied by Messrs. I.C.I. Ltd., Jealott's Hill. All simple electrolytes used were of Analar quality.

RESULTS AND DISCUSSION

The variation of the times t_1 and t_2 (defined above) with pH and concentration of MCPA and IAA are shown in Tables I and II.

TABLE I

MCPA, pK 3·14

Total concentration	Bulk pH	t_1	t_2	Concentration $\times\, t_1$
222 mg./l.	3·30	1·5 min.	3 min.	333
74	3·25	4·5	2·5	330
55	3·21	7·0	2·5	385
37	3·25	8·5	2·5	314
222	4·30	4·5	24	1,000
148	4·35	9·0	28	1,333
40	4·30	25	25	1,000
222	4·10	3	4·5	666
444	4·23	2	9	888
220	4·50	7	$\sim$ 100	1,540
444	5·12	6	∞	2,664
446	5·86	40	∞	17,800
446	6·90	120	∞	53,400

TABLE II

IAA, pK 4·75

Total concentration	Bulk pH	t_1	t_2	Concentration $\times\, t_1$
200 mg./l.	3·95	5·5 min.	17 min.	1,100
100	3·95	10	18	1,000
50	3·9	27	15	1,300
25	3·95	42	17	1,050
10	3·95	85	20	850
200	3·10	4·5	6	900
200	3·6	5	13	1,000
200	4·2	6	38	1,200
200	4·4	6	$\sim$ 120	1,200

From Table I it will be seen that when the pH is kept constant, and the total concentration of substance varied, t_2 remains effectively constant, as does the value of (concentration $\times\, t_1$). For a different constant pH value, t_2 and (concentration $\times\, t_1$) have new constant values.

The time t_1

The Henderson equation

$$\mathrm{pH} = \mathrm{pK} + \log_{10} \frac{\text{conc. of ions}}{\text{conc. of undissoc. molecules}}$$

can be used to determine the concentration of undissociated molecules present in the external solution, but it must be remembered that it is the surface pH value which determines the relative concentrations of molecules and ions in the immediate vicinity of the surface of the membrane.

If, in the case of *Nitella*, the $pH_{surface}$ is about 1·1 units lower than the pH_{bulk}, then it is possible to evaluate the concentration of molecules near the surface for each case in Tables I and II. The results are illustrated in Table III for MCPA, and represented graphically in Fig. 2, which shows a plot of surface concentration of molecules against time t_1 for both MCPA and IAA.

TABLE III

Total conc. of MCPA	Surface pH	Surface conc. of molecules (a)	t_1	$a\times t_1$
222 mg./l.	2·20	196 mg./l.	1·5 min.	295
74	2·15	66	4·5	295
55	2·11	49	7	343
37	2·15	34	8·5	287
222	3·20	60	4·5	270
148	3·25	37	9·0	333
40	3·20	12	25	300
222	3·00	110	3	330
444	3·13	175	2	350
220	3·46	44	7	308
444	4·02	44	6	264
446	4·76	9	40	360
446	5·80	3	120	360

It must be pointed out that only by taking the pH difference at the surface to be about 1·1 units do the values of $(a\times t_1)$ remain constant throughout the whole table, and all the points lie on the smooth curves in Fig. 2.

It may be suggested that the curves represent a simple diffusion process, and that the cessation of cytoplasmic streaming is due to the same amount of growth substance having passed into the cytoplasm. In this case, from Fig. 2 it would appear that the rate of entry of the undissociated molecule is proportional only to concentration of molecules in the external solution, right at the surface of the membrane. The Fick diffusion law then reduces to

$$dc/dt = Ka,$$

where dc/dt = rate of entry of molecules, a = surface concentration of molecules, K = diffusion constant.

This on integration gives

$$t = c/Ka, \text{ or the product } a\times t = \text{constant.}$$

c would here be the amount of substance per unit volume which must pass into the cytoplasm in order to stop streaming.

If the curves in Fig. 2 are in fact an expression of the Fick law, then it must be supposed that the concentration of free growth substance in solution in the cytoplasm is always negligible. This might be possible were the substance removed from solution by adsorption on to suitable sites as soon as it passes

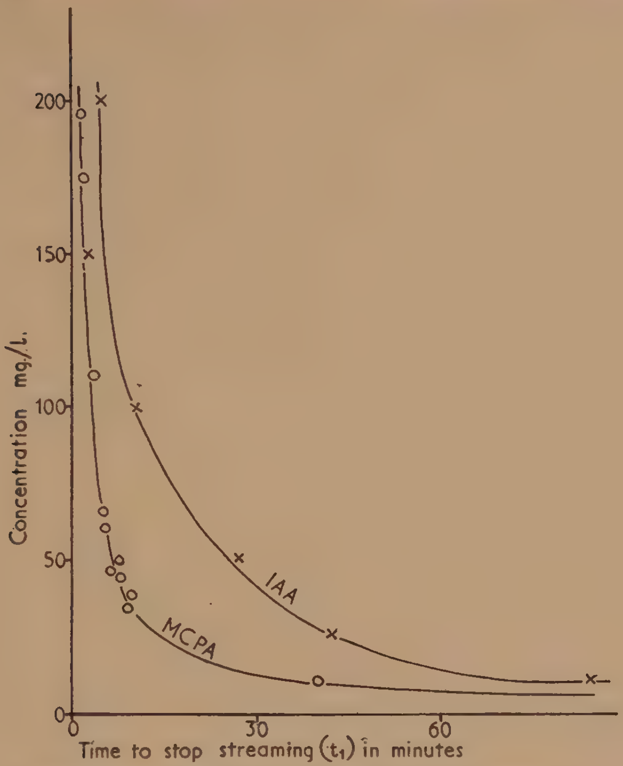


FIG. 2. Surface concentration of molecules of MCPA and IAA against time t_1. Both curves are rectangular hyperbolas fitting $a \times t_1 =$ constant.

through the membrane, and it would follow that when all or a certain fraction of the sites are filled, cytoplasmic streaming stops. However, it must be noted that if a cell is replaced in its culture medium before the 'flood' occurs, cytoplasm streaming recommences and returns to its normal rate. Thus, either the postulated adsorption of growth substance within the cytoplasm must be reversible or the cell must be capable of breaking down the growth substance, thereby allowing the normal metabolic processes to continue.

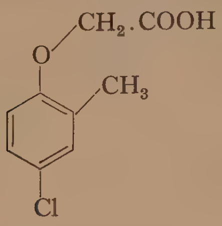
In the case of IAA, the value of $(a \times t_1)$ and hence c/K is about 1,000, whereas for MCPA the value is about 300. If it may be suggested that, because the two substances contain the same active groupings, the same number of molecules of each substance must enter the cytoplasm in order to stop streaming, then the ratio of the two diffusion constants may be obtained, thus:

$$\frac{c/K_{\mathrm{IAA}}}{c/K_{\mathrm{MCPA}}} = \frac{1000/M_{\mathrm{IAA}}}{300/M_{\mathrm{MCPA}}},$$

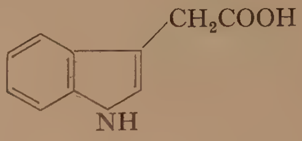
where $M_{\mathrm{IAA,\,MCPA}}$ are the molecular weights, 175 and 223 respectively, which gives

$$\frac{K_{\mathrm{MCPA}}}{K_{\mathrm{IAA}}} \simeq 3{\cdot}5.$$

In view of the relative structures and dimensions of the two molecules



MCPA



IAA

it might be expected that MCPA would penetrate through the membrane more readily than IAA. A further measure of support for this view can be obtained from measurements of the ionic mobilities of the two ions in aqueous solution. These were measured, and the ratio of the ionic mobilities at infinite dilutions was found to be

$$\frac{\lambda^{\circ}_{\mathrm{MCPA}}}{\lambda^{\circ}_{\mathrm{IAA}}} \simeq 2.$$

Albaum, Kaiser, and Nestler (1937) have determined a value for the diffusion coefficient of IAA passing from the external solution, through the cytoplasm, into the vacuolar sap of *Nitella*. Using 0·003 Molar solutions, their value was 0·0428 moles. min.$^{-1}$, but it is doubtful if the coefficient for diffusion across the membrane between the external solution and the cytoplasm (K_{IAA} above) has this value. If this coefficient has, in fact, a value of the order of 0·05 moles. min.$^{-1}$, then some estimate of the value c, the amount required to stop streaming, can be made. The value obtained is about 0·25 moles/(litre of cytoplasm), and since an average sized *Nitella* cell probably contains only 10^{-5} ml. of cytoplasm, the actual amount of IAA required is $0{\cdot}25 \times 10^{-8}$ moles. This would mean that 10^{15} molecules of IAA must pass into the cytoplasm to stop streaming, and it is possible that, on the hypothesis presented above, there is a relation between this figure and the number of suitable adsorption sites present within the cytoplasm.

The importance of the negatively charged membrane

It has already been suggested that it is the pH at the surface of the membrane which determines the relative concentrations of molecules and ions in the vicinity of the cell membrane. Hartley and Roe (1940) have put forward a simple relationship between $\mathrm{pH}_{\mathrm{surface}}$, $\mathrm{pH}_{\mathrm{bulk}}$, and the electrokinetic or ζ potential of the system.

$$\mathrm{pH}_s = \mathrm{pH}_b + \frac{e\zeta}{2{\cdot}3kT},$$

where e = electronic charge, k = Boltzmann constant, T = absolute temp.

In the case of *Nitella*, and similar systems, it is convenient to follow the treatments of Gouy and Stern (Verwey and Van Overbeek, 1948; Kruyt, 1952) and replace ζ by a potential ψ at a distance x from the charged layer in the membrane where the potential is ψ_0. The value of ψ will then determine the difference between $pH_{surface}$ and pH_{bulk}, which will in turn determine the ionization of the growth substance in the vicinity of the membrane.

According to the theories of Gouy and Stern, the ψ potentials at any charged surface should decrease with increasing salt concentration in the adjacent solution, and this has been confirmed experimentally in many cases.

A series of experiments was carried out on *Nitella* with MCPA in order to determine the change in pH difference at the surface of the membrane with increasing concentrations of sodium chloride. It was found that $pH_{surface}$ approached pH_{bulk} as the concentration of NaCl was raised. The results are expressed in Table IV.

TABLE IV

Conc. of MCPA	Total $[Na^+]$	pH_{bulk}	t_1	Conc. of molecules from Fig. 2	$pH_{surface}$	$pH_b - pH_s$
40 mg./l.	0·0002 N	4·40	25 min.	12 mg./l.	3·29	1·11
223	0·001 N	4·10	2·5	130	3·01	1·09
223	0·002 N	4·36	3·5	83	3·37	0·99
223	0·005 N	4·24	5·0	60	3·42	0·82
223	0·011 N	4·08	4·5	70	3·34	0·74

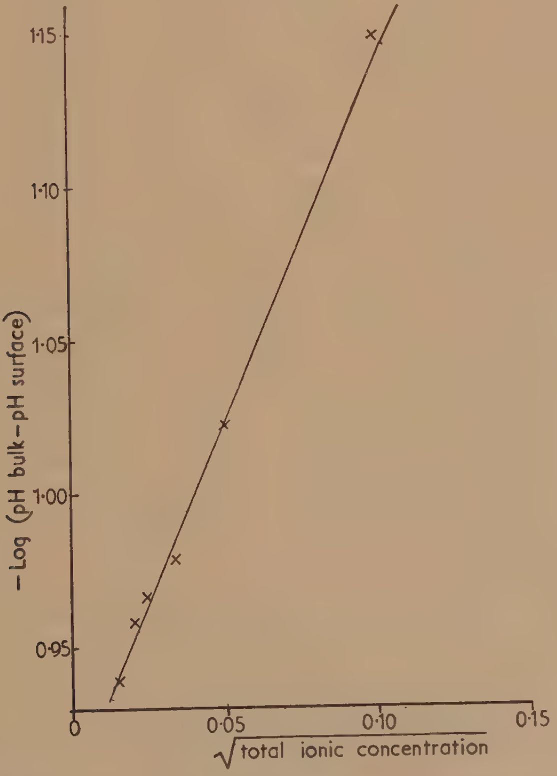


FIG. 3. $-\log_{10}(pH_{bulk} - pH_{surface})$ against $\sqrt{}$(total ionic concentration) giving the relation expected from $A\sqrt{c}.x = -\log_e \psi/\psi_0$.

For small ψ potentials the Gouy expression relating ψ with the total ionic concentration can be reduced to the approximate equation

$$A\sqrt{c}.x = \log_e\psi_0 - \log_e\psi,$$

where c = total ionic concentration, A = constant.

Over the concentration range 0·0002–0·01 N, it may be assumed that ψ changes to a much greater extent than ψ_0, and hence there should be a linear relation between $\log_e \psi$ and $\sqrt{c}$. If the pH differences (last column, Table IV) are taken as a measure of ψ, and $\log_{10}(pH_b - pH_s)$ plotted against $\sqrt{c}$, a straight line is obtained as shown in Fig. 3.

The validity of the simplified Gouy expression in this case is questionable since the ψ potential terms have little significance. However, the result expressed in Fig. 3 does lend support to the view that the charged surface of the cell membrane plays a considerable part in determining the dissociation of weak acids and bases in its vicinity.

The use of strong buffer solutions, as described by many workers, does tend to swamp out this membrane effect, but with *Nitella* even a solution 0·011 N in strength leaves a pH difference of 0·74 units at the surface.

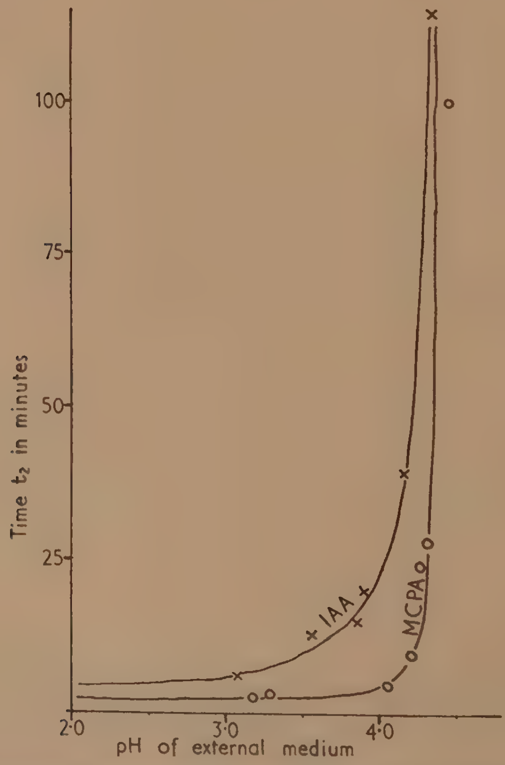


FIG. 4. pH of external medium against time interval between cessation of cytoplasmic streaming and the observed 'flood', i.e. time t_2.

The time t_2

From Tables I and II it will be noticed that the time t_2 is completely independent of the total concentration of growth substance in the external medium, but depends only on its pH value. The curves of t_2 against pH for MCPA and IAA are shown in Fig. 4, and it is seen that they approach an asymptote at pH 4·4, above which no 'flood' has been observed.

It has been noted that, after the 'flood' has been observed, the cell is apparently dead, and yet the 'flood' is accompanied by a profound (350 per cent.) increase in membrane resistance. Now it has been shown by Blinks (1930), Osterhout (1931), and Cole (1940) that injury and death of a cell are both usually accompanied by a marked *fall* in membrane resistance; so it would appear that the plant growth substances used here are unique in that their toxic effect on *Nitella* produces the reverse resistance effect.

CONCLUSIONS

1. When a *Nitella* cell is placed in a solution of certain plant growth substances, after a time (t_1 min.) cytoplasmic streaming stops. After a further time (t_2 min.) a flood of cytoplasmic particles is seen streaming to one end of the cell, this process being accompanied by a profound increase in the cell membrane resistance and death of the cell.

2. The time t_1 depends only on the concentration of undissociated molecules of growth substance in the vicinity of the surface of the membrane. It would appear that this concentration depends on the pH at the surface, which, in turn, is governed by the bulk pH of the solution and the ψ potential at the surface. The latter varies with the total ionic concentration in the adjacent bulk phase, $\mathrm{pH}_{\mathrm{surface}}$ approaching $\mathrm{pH}_{\mathrm{bulk}}$ as the ionic concentration increases.

3. If the assumption be made that the cessation of cytoplasmic streaming is a result of a certain amount of growth substance having penetrated into the cytoplasm, a simple Fick diffusion process would account for the results obtained. In this case it is necessary to postulate that the growth substance is removed from solution upon entry into the cytoplasm. One possible view is that the substance may be adsorbed on to certain sites, e.g. the keto-amido-linkage of lipo-protein structures, in the manner described by Brian and Rideal (1952).

4. On the above hypothesis, a ratio of the diffusion constants for the undissociated molecules of IAA and MCPA, penetrating into the cytoplasm of *Nitella*, may be evaluated. The ratio is of the same order as the ratio of the respective ionic mobilities at infinite dilution.

5. The time t_2 depends only on the pH of the external solution. The toxic effect of the growth substances used is accompanied by a marked *increase* in membrane resistance, this being the reverse of the resistance effect normally accompanying death of the cell.

ACKNOWLEDGEMENTS

The author would like to express his thanks to Professor Sir Eric Rideal, F.R.S., under whose supervision the work was carried out, and to Professor T. A. Bennet-Clark, F.R.S., for many helpful discussions. The author also wishes to thank the Agricultural Research Council for generous financial support.

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Some Quantitative Changes in the Fat Metabolism of Germinating Lettuce Seeds and their Inhibition by Coumarin

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SUMMARY

The fat reserve of lettuce seeds of two varieties, 'Grand Rapids' and 'Progress', was examined. It was found to be an unsaturated lipid, closely resembling sunflower-seed oil. The changes in the fat content with time and under treatment with thiourea and with coumarin were studied, as were the changes in the acidity of the fat. No free volatile saturated acids were detected. On hydrolysis the seeds yield one such acid whose identity was not finally established and whose origin is uncertain, it not being certain that it is connected directly with fat metabolism.

THIS investigation is part of a more extensive research programme carried out in these laboratories. The general aim of the research is to elucidate the mechanism of action of coumarin as a germination inhibitor and thiourea as a substance breaking the dark dormancy of lettuce seed. The effect of coumarin on various enzymes in crude extracts *in vitro* and *in vivo* has already been described (Mayer, 1954; Poljakoff-Mayber, 1952*a*, *b*, 1953*a*, *b*, *c*). Various other aspects of metabolism during germination and its inhibition have also been studied (Levari, 1953; Levari, Mayer, and Evenari, 1952; Mayer, 1953; Poljakoff-Mayber, 1952*a*).

Coumarin was found to inhibit the lipolytic activity of crude enzyme extracts *in vitro* (Poljakoff-Mayber, 1953*b*). In lettuce seeds germinated in coumarin it was found that there was no decrease in fat content, the fat apparently not being utilized. In water and thiourea, however, the fat content decreased during germination. Coumarin germinated seeds also show no increase in reducing sugars, as opposed to the process in water and in thiourea. It appeared, therefore, that coumarin directly affected the fat metabolism of lettuce seeds. In continuation of this work it was, therefore, decided to study the changes in the fatty acids taking place in the seeds during germination and its inhibition. At the same time some of the properties of the fat, which appears as storage material, were studied.

MATERIAL AND METHODS

Two varieties of lettuce seeds were used. The light sensitive variety 'Grand Rapids' and the light indifferent variety 'Progress'.[1] The fat was extracted

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[1] Our thanks are due to Messrs. Pieters-Wheeler Seed Co., U.S.A., who kindly supplied the seeds.

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with ether in the cold. The ether was evaporated *in vacuo* and the residue taken up in light petroleum ether. The extract was filtered and evaporated *in vacuo* to constant weight.

The refractive index of the fat, its saponification number, and iodine number were determined by the conventional methods. Free fat acidity was measured by the method described by the A.O.A.C. (1945).

The total volatile fatty acid content of the saponified fat was determined by the method described by Triebold (1947) for the determination of Reichert-Meissl and Płolenske numbers. The soluble and insoluble fatty acids were not separated.

Paper chromatograms were run as described by Reid and Lederer (1951) in order to identify the saturated fatty acids C_2—C_7.

RESULTS

The characteristics of the fat reserve of the lettuce varieties 'Progress' and 'Grand Rapids' are given in Table I.

TABLE I

Some characteristics of the lipid of lettuce seeds

	Variety	
	'Progress'	'Grand Rapids'
Fat content, % dry weight	36·0 s.d. 2·3	38·8 s.d. 3·1
Refractive index	1·476	1·475
Saponification No.	189–90	181–9
Iodine No.	125–7	125–31

Changes in the fat content of seeds variety 'Progress' were measured, the results being summarized in Fig. 1*a*. It must be pointed out that in 10 mg. per cent. coumarin the germination of the seeds is low (30–40 per cent.) and in addition it is retarded. Seeds in 125 mg. per cent. thiourea germinate as well and as quickly as seeds in water. Typical changes in the sugar content of seeds germinated in water are given for comparison in Fig. 1*b*. It is worth noting that in thiourea there is a characteristic development of a yellow pigment in the root-tip of the emerging root. Its identity has not yet been established.

As the total fat content did not point to any very significant effect of thiourea on the process, the free total acidity of the fat in seeds of variety 'Progress' was studied. The results are shown in Fig. 2. It will be seen that here the effect of thiourea is very marked, whereas the seeds in coumarin behaved very much like those in water. The volatile fatty acid content of the seeds after saponification was also studied. The results are given in Fig. 3, where the changes of the volatile acids in seeds variety 'Progress' under different treatments are given, and in Fig. 4, where the effect of light and darkness on the volatile fatty acids of variety 'Grand Rapids' is given. An attempt was also made to identify the volatile saturated fatty acids. Neither dry seeds nor germinated ones contained any free volatile saturated fatty acids. After saponification, however, analysis by paper chromatography showed one spot and one spot only at all stages of germination and under all treatments, whether in water, thiourea, or coumarin. This spot had an R_F value of 0·07–0·10, which corresponds to formic acid in control chromatograms, but which may equally well be due to either acetic or lactic acids. These three acids are not separable by the method used. In view of the very small amounts of the acid present, attempts to identify it by chemical means were unsuccessful.

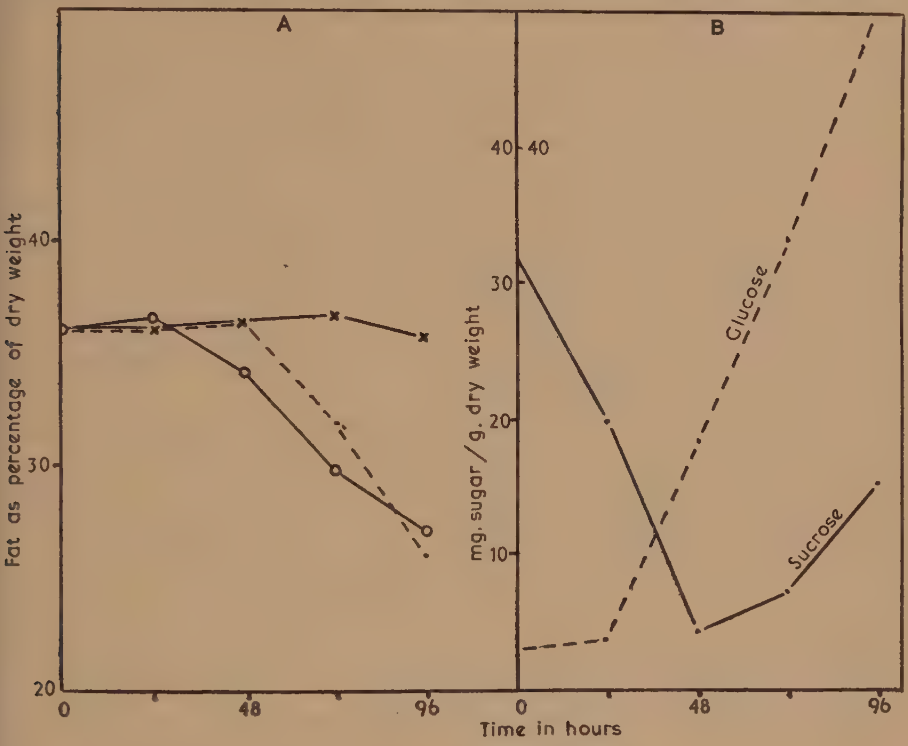

FIG. 1 *a*. Changes in fat content of 'Progress' seeds during germination: ○——○——○ seeds germinated in water; ×——×——× seeds germinated in 10 mg. % coumarin; (– – – – –) seeds germinated in 125 mg. % thiourea. *b*. Changes in the sugar content of 'Progress' seeds germinated in water.

DISCUSSION

The properties of the seed fat in both varieties of seeds indicate that there is no essential difference between their fat reserves. Moreover, a comparison of the properties of the lipid show it to be very similar to that of sunflower-seed oil. The lipid is highly unsaturated, although not as highly as linseed oil. It may therefore be assumed that the unsaturated fatty acid content of the seeds is rather high.

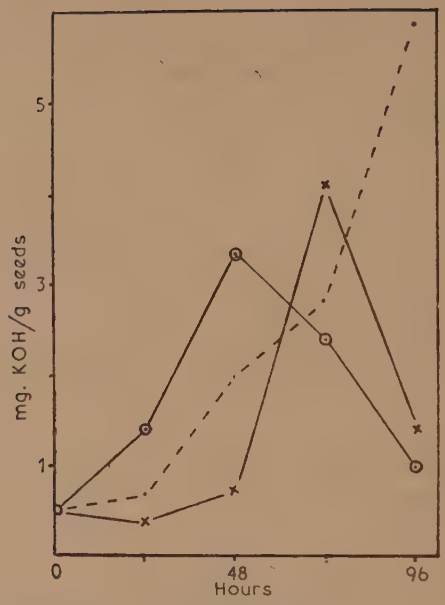

FIG. 2. Changes in free fatty acid content of seeds variety 'Progress' during germination: (○——○——○) seeds germinated in water; (×——×——×) seeds germinated in 10 mg. % coumarin; (-----) seeds germinated in 125 mg. % thiourea.

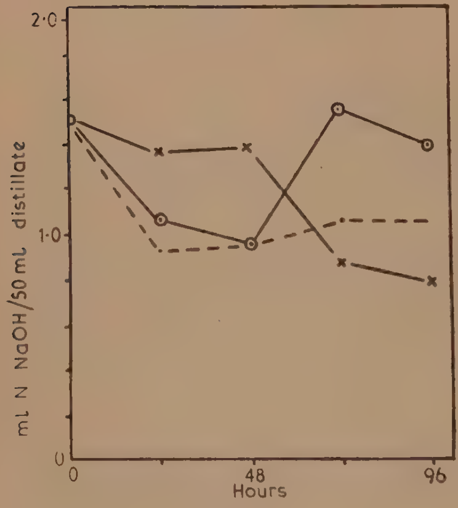

FIG. 3.

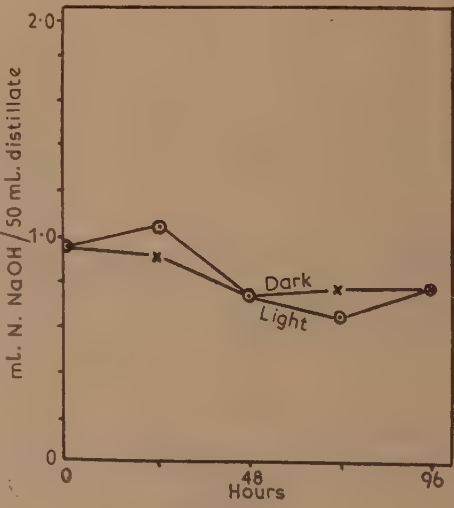

FIG. 4.

FIG. 3. Changes in the volatile fatty acid content of the saponified fat during the germination of lettuce seeds variety 'Progress'. Conventions as in Fig. 2.

FIG. 4. Changes in the volatile fatty acid content of the saponified fat during the germination of 'Grand Rapids' in light and in darkness.

The fat content as percentage of dry weight of the two varieties of seed is the same, the differences being non-significant. On the other hand, the breakdown of fat in the two varieties differs appreciably, starting earlier in 'Progress' (cf. Fig. 1, Poljakoff-Mayber, 1952*a*).

The hydrolysis of fat is blocked in both varieties by coumarin, whereas thiourea shows no effect, except a slight delay in the onset of hydrolysis. It can therefore be assumed that the effect of both coumarin and thiourea on the two varieties of seed is identical as far as this aspect of metabolism is concerned. The free fatty acids of the seed reach a maximum after 48 hours of germination in water (Fig. 2). This period corresponds with a minimum sucrose content of the seeds (Fig. 1*b*). At the same time glucose synthesis in the seeds begins after 24 hours and then rises more or less linearly, parallel to fat hydrolysis. It is interesting to note that Levari (1953) has shown that lettuce seeds variety 'Progress' have an initial R.Q. of approximately 0·8 and that a 20 per cent. increase in Q_{O_2} occurs at about the 24th hour. These facts of lower R.Q., increased oxygen consumption, intensive fat breakdown, and the changes in sugar content suggest a close interrelationship between the various storage materials which is much more marked during early stages of germination in the variety 'Progress' than in the variety 'Grand Rapids', where the R.Q. is 1 or very near to it and the amount of oxygen consumed is very near to that needed for complete oxidation of amount of sugar disappearing in the first 24 hours of germination.

Further, it is clear that the fatty acids involved are not volatile saturated ones, C_2—C_7, as these were not detected free in the seeds. Coumarin prevents fat hydrolysis but not the utilization of the sucrose reserve present in the seeds (Poljakoff-Mayber, 1952*a*). Thiourea must act by an entirely different mechanism, as its action causes a continuous fatty acid accumulation (Fig. 2). This might be due to a direct acceleration of the hydrolysis of fat to fatty acids.

The experiments summarized in Figs. 3 and 4 are difficult to interpret. Fig. 4 shows that there is no appreciable difference between volatile fatty acids content of the saponified fats of 'Grand Rapids' seeds germinated in light and in darkness. The fatty acids of the saponified fat decrease somewhat with time, but by no means as rapidly as does the fat content itself.

In seeds variety 'Progress' the total acidity of the fat rises until the 48th hour and then falls, while the volatile fatty acids content of the fat decreases till the 48th hour and then shows a marked rise. The final decrease only occurs after 72 hours. Moreover, in coumarin the volatile fatty acids fall steadily. It almost appears as if in coumarin certain easily hydrolysable volatile acids can be utilized but that the fat cannot be completely broken down.

Thiourea, again, shows an entirely different method of action. Here the total fatty acids continuously increase, while the volatile fatty acids remain in a steady state after the first 24 hours. It is difficult to explain this unless it is assumed that the total acidity is due to the direct hydrolysis of fat, while those volatile acids which are liberated on saponification of the seeds are of various origins. This is not at all unlikely as in the method involved, hydrolysis of the entire seed, other esters than those found in the fats could easily be decomposed. This is also indicated by the fact that the saturated volatile fatty acid, although not finally identified, is probably acetic acid, and it is difficult to account for its appearance merely by hydrolysis of fat. Acetic acid could, however, easily occur as a product of intermediary metabolism of fats.

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The Growth-time Relationships of the Auxin-induced Growth in *Avena* Coleoptile Sections[1]

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SUMMARY

1. The growth rate of *Avena* coleoptile sections in the presence of indoleacetic acid (IAA) is constant with time over a wide range of time intervals and IAA concentrations.

2. Constancy of growth rate is dependent upon the maintenance of constant conditions in which the concentration of IAA available to the section remains the chief factor limiting growth rate.

3. Control of the pH of the medium in which the sections are grown is essential to the maintenance of constant growth rate, particularly in the presence of high concentrations of IAA.

4. The lag period in establishment of steady growth rate by sections in the presence of IAA is less than 10 minutes and is not detectable by present methods of measurement.

INTRODUCTION

It is known that the elongation of excised sections of the *Avena* coleoptile is dependent upon auxin and that rapid growth of such sections occurs when they are placed on solutions of an auxin such as indoleacetic acid (IAA) (Bonner, 1933). It has also been shown that the *Avena* coleoptile section-auxin system may be treated by the methods of enzyme kinetics (Foster, McRae, and Bonner, 1952; McRae and Bonner, 1952; McRae and Bonner, 1953; McRae, Foster, and Bonner, 1953). Growth rate of the section increases hyperbolically with increasing auxin concentration just as does rate of an enzymatic reaction with increasing substrate concentration. In the kinetic analysis of the *Avena* section response to auxin, growth velocity is measured as a function of substrate (IAA) concentration. It is important to this treatment that the growth rate measured be constant with time; be a true initial reaction velocity. Data have been presented to define the conditions under which growth of *Avena* coleoptile sections is linear with time, and the kinetic treatment of *Avena* section growth has been applied to data obtained under these conditions. Bennet-Clark and Kefford (1954) have presented data in this journal from which they draw the conclusion that the growth rate of excised coleoptile sections is not constant with time over any considerable range of times or auxin concentrations, and that the kinetic treatment of auxin-induced growth is thereby invalid.

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There are doubtless many experimental conditions under which the extension of *Avena* coleoptile sections is not linear with time for any considerable period. Bennet-Clark and Kefford have not used the experimental conditions which we have found specifically suitable for kinetic analysis, but have changed these conditions in at least three ways which in our opinion are important. These are: (1) the use of coleoptiles 1·5 to 2 cm. in length rather than 2·75 to 3·25 cm. in length as recommended by McRae and Bonner (1953); (2) lack of pH control; (3) lack of potassium ions. It will be shown below that the alterations in experimental conditions made by Bennet-Clark and Kefford are important ones; that they result in non-constancy of *Avena* coleoptile section growth rate with time, and that the conclusions of Bennet-Clark and Kefford are invalid.

METHODS

Avena plants of the variety of Siegeshafer (obtained from Svalöf, Sweden) were grown in vermiculite contained in stainless steel trays and watered with distilled water as described by McRae and Bonner (1953). The growing of the plants as well as all operations on the plants was conducted in a standard *Avena* room at a temperature of 25° C., a humidity of 90 per cent., and under continuous low intensity red light with occasional orange light. After 82–84 hours a selection was made of those plants whose coleoptiles were 2·75 to 3·25 cm. in length. From each coleoptile a single 5·0-mm. section was cut 2 to 3 mm. from the tip. Primary leaves were not removed from the sections since growth rate of the coleoptile is unaffected by their presence under our conditions. The sections were pooled, randomized, and distributed in lots of 20 to Petri dishes containing 20 ml. of solution. It should be noted that Bennet-Clark and Kefford (1954) use 15 sections, each 3 mm. long, in 5 ml. of solution. It is possible that the greater ratio of tissue to solution used by them may be of some importance. The basal medium consisted of pyrex redistilled water, 3 per cent. sucrose, and 0·0025 M. K maleate buffer (pH 4·5). Auxin, IAA except where noted, was added to this basal medium as indicated. All auxin solutions were prepared in pyrex redistilled water and adjusted to pH 4·5 with KOH. The sections were incubated from 2 to 18 hours and length measurements made under a microscope fitted with a stage micrometer. Each measurement of growth at each successive time interval was done on a separate lot of 20 sections. Sections were not measured and then returned to the nutrient for further incubation, as done by Bennet-Clark and Kefford. The precision of growth measurements under the present conditions as well as day-to-day variations to be expected have been analysed by McRae and Bonner (1953).

EXPERIMENTAL RESULTS

In an earlier paper it has been shown that growth rate of *Avena* coleoptile sections is constant with time over the period zero to 18 hours under the present conditions and with an auxin concentration which yields approximately maximal growth rate, namely 5·7 × 10^{-6} M. (McRae and Bonner, 1953; McRae, 1953). Bennet-Clark and Kefford (1954) suggest that similar relations may not hold true for other auxin concentrations. The data of Fig. 1 show, however, that growth rate is linear with time up to 18 hours for auxin concentrations which are lower than optimal as well as for auxin concentrations which are higher than optimal, as, for example, 5 × 10^{-4} M. The conditions chosen for our experiments are then, in fact, such that growth rate is constant over a considerable period of time and over a wide range of auxin concentrations. The data of Fig. 1 are plotted as a function of auxin concentration in Fig. 2. It may be seen that the growth velocity-auxin concentration

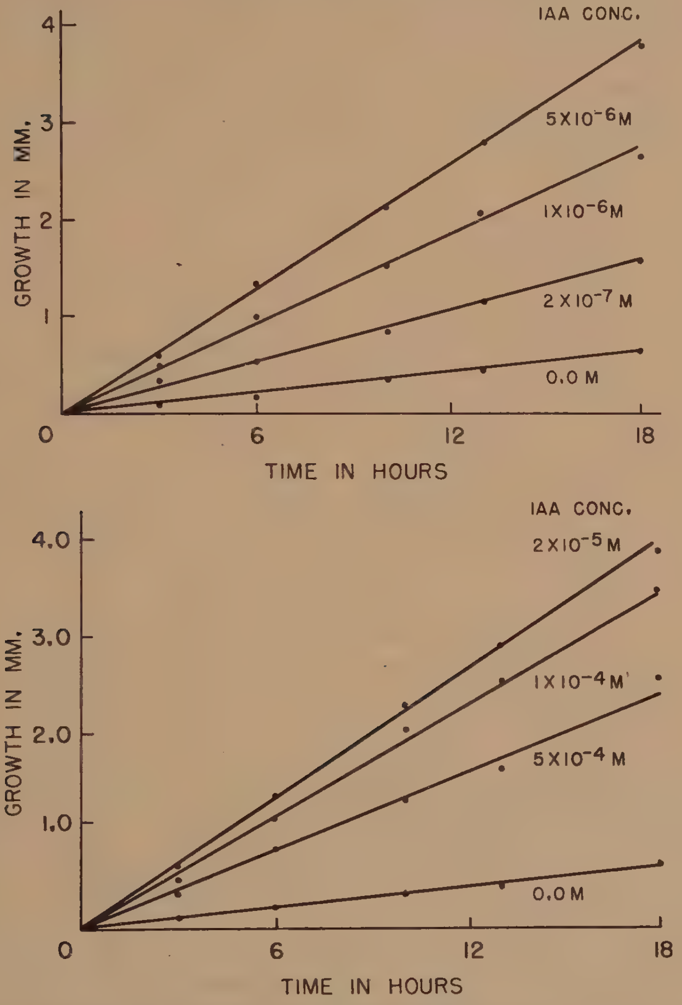

FIG. 1. Progress curves for growth of *Avena* coleoptile sections. Above: Sub-optimal auxin (IAA) concentrations. Below: Supra-optimal auxin (IAA) concentrations.

curves obtained at varying time intervals from 3 to 18 hours form a family of curves which increase regularly with increasing time of incubation of the sections. These relations hold true over the entire range of auxin concentrations to which the *Avena* coleoptile is responsive.

Bennet-Clark and Kefford have been particularly concerned with growth rate-time relations of *Avena* sections in the presence of high auxin concentrations, concentrations great enough to cause appreciable auxin-induced growth inhibition. They suggest that auxin-induced growth inhibition may not be due to a low but constant growth rate, but that it may rather be due to an initial rapid growth rate which quickly dies away as the tissue is damaged in some fashion by high auxin concentration. That the suggestion of Bennet-Clark and Kefford is not applicable to our experiments has been shown in Fig. 1. It may, in addition, be shown directly that *Avena* coleoptile section tissue is not damaged by exposure to high auxin concentration. For this experiment sections were first incubated in auxin of a concentration (5×10^{-4} M.) which permits of a growth rate of approximately one-half maximal. After 4 hours in this solution, sections were removed either to auxin-free basal medium or to a solution containing auxin in a 100-fold lower concentration, a concentration approximately optimal for *Avena* coleoptile section growth. The results of such an experiment are presented in Fig. 3. Sections in 5×10^{-4} M. auxin solution grow at a constant rate over the 12-hour interval considered. The same is true for sections in 5×10^{-6} M. auxin solution. Sections removed from the higher to the lower concentration increase in growth rate. In approximately 2 hours they have become equilibrated to the lower concentration and grow at the same rate as sections which have been continuously in such a solution. The sections have not, therefore, been damaged. It may be noted that the equilibration

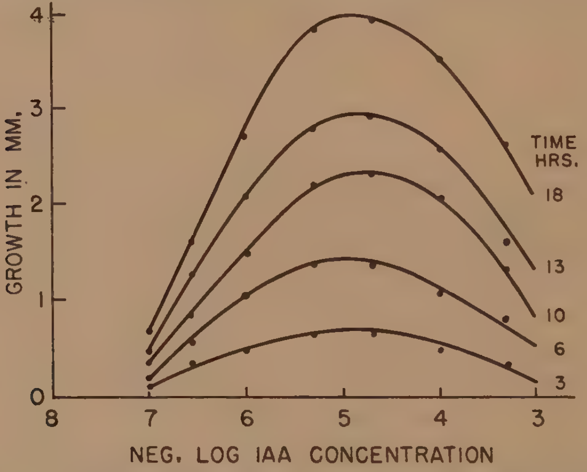

FIG. 2. Coleoptile section growth *v.* auxin (IAA) concentration for various periods of incubation. The top curve is for an 18-hour incubation. Successively lower curves are for 13-, 10-, 6-, and 3-hour periods.

time for the experiment of Fig. 3, approximately 2 hours, is longer than the equilibration time for sections which are merely moved from an auxin solution of low concentration to a solution of a second auxin, such as 2:4-D, also of low concentration (Foster, McRae, and Bonner, 1952).

The progress curves for *Avena* section growth obtained by Bennet-Clark and Kefford for high auxin concentrations are characterized by a rapid decline in growth rate. It has been shown above that our experimental conditions yield progress curves which are linear with time for 18 hours. The failure of Bennet-Clark and Kefford to duplicate our results is due to the fact that they failed to include potassium ions and buffer in the basal nutrient. That this is so is shown in Fig. 4. For the experiment of Fig. 4, sections were grown in solutions 5×10^{-4} M. in IAA. One set of sections received the basal medium of McRae and Bonner (1953) including 0·0025 M. K maleate buffer, pH 4·5. The IAA used in this part of the experiment was also titrated to pH 4·5 with KOH. A second set of sections were cultured in basal medium lacking buffer. The IAA used in this part of the experiment was, following the technique of Bennet-Clark and Kefford, not neutralized with KOH. The data of Fig. 4

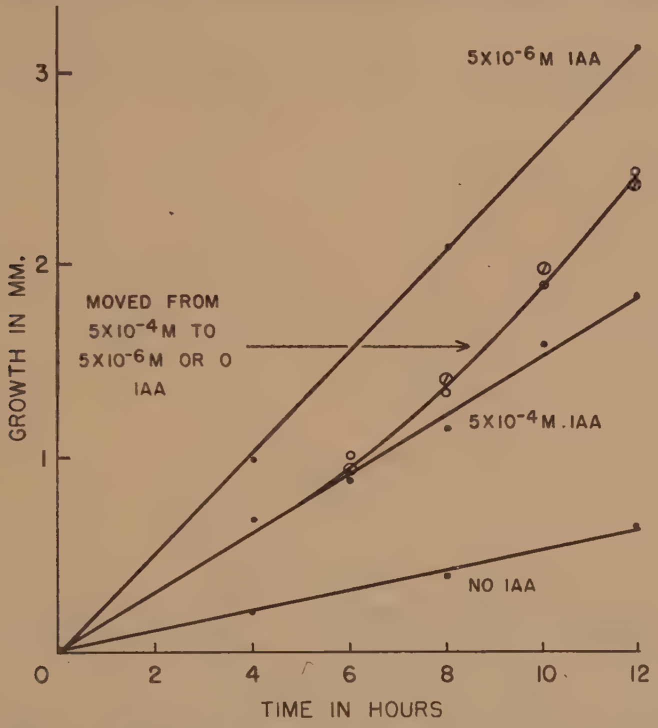

FIG. 3. Reversibility of the high auxin concentration-induced growth inhibition of coleoptile sections. Straight lines through solid dots, from tip to bottom; IAA concentrations of 5×10^{-6} M., 5×10^{-4} M., and none. At 4 hours, sections were removed from 5×10^{-4} M. IAA to IAA concentrations of none (o) or of 5×10^{-6} M. (⦶).

show clearly that growth rate is maintained at a steady rate for the 18 hours of the experiment only if buffer is present in the medium. In the absence of added buffer, growth rate drops to zero after 8 hours, a result generally similar to that found by Bennet-Clark and Kefford.

When *Avena* coleoptiles are decapitated, as for the standard *Avena* curvature test, the growth rate of the stump drops to a low value after 2–3 hours and then increases gradually. This behaviour is due to the regeneration by the apical portion of the stump of the ability to produce auxin. The response is known as the regeneration of the physiological tip (Went and Thimann, 1937). In the initial work with growth of excised coleoptile sections it was found that such regeneration of the physiological tip does not occur with sections immersed in solution. The growth rate of sections without added auxin remains constant for a period which depends upon many factors, and then gradually sinks to a low level (Bonner, 1933). Similar results have been reported by Schneider (1938), Bonner (1949), McRae (1953), and others. Bennet-Clark and Kefford conclude, however, that the extension-time curve for control sections possesses a sigmoid shape. They suggest that the endogenous growth of coleoptile sections as well as growth in response to added auxin may possess a lag period as does the auxin-induced growth of storage tissue. We do not find any lag period by our method of measurement under our conditions as has been shown in Figs. 1 and 3. The data of Fig. 5 give further information concerning the time needed to establish steady state

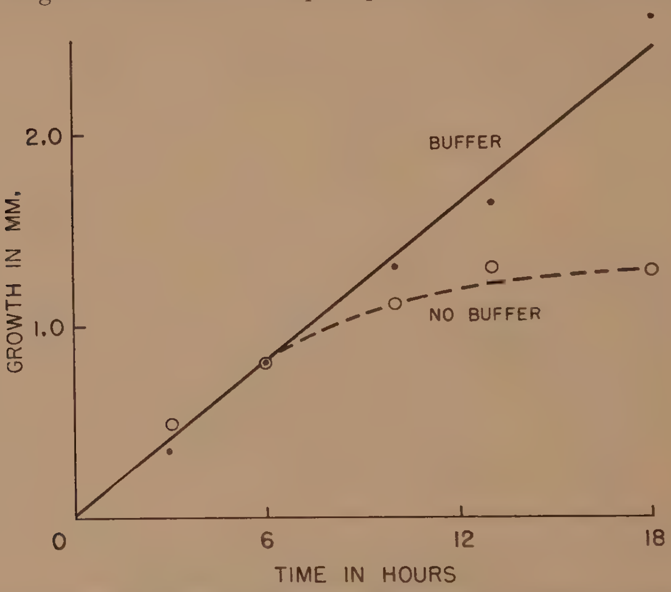

FIG. 4. Effect of potassium maleate buffer on progress curves for growth of coleoptile sections in the presence of a high (5 × 10⁻⁴ M.) concentration of IAA. Upper curve (solid), in presence of $2{\cdot}5 \times 10^{-3}$ M. potassium maleate buffer, pH 4·5, IAA titrated to pH 4·5 (conditions of Foster, McRae, and Bonner, 1952). Lower curve (dotted), no buffer. IAA not neutralized (conditions of Bennet-Clark and Kefford, 1954).

growth rates in response to added auxin. For this experiment growth measurements were made at shorter intervals than in the previous experiments, namely every 10–20 minutes rather than every 2–4 hours. It is again apparent that, with the varied auxin concentrations used, growth is linear with time from zero time onward. It may be concluded that any lag period in initial equilibration of the section with auxin is less than approximately 10 minutes under our conditions. It would conceivably be of interest to discover which of the experimental procedures of Bennet-Clark and Kefford produces the slightly sigmoid shape of their extension-time curves. It cannot be attributed to the absence of buffer or potassium ions, since the data of Bonner (1933) and of Schneider (1938) which show no lag period were also obtained in buffer and potassium ion-free medium. It appears possible that the slight apparent sigmoid shape of the extension-time curves obtained by Bennet-Clark and Kefford may be an artifact of their method of measurement. The growth increments at the shorter time intervals are small, particularly in the case of the control (no added auxin) series, and of the same order of magnitude as the smallest units used in measuring the growth of the sections.

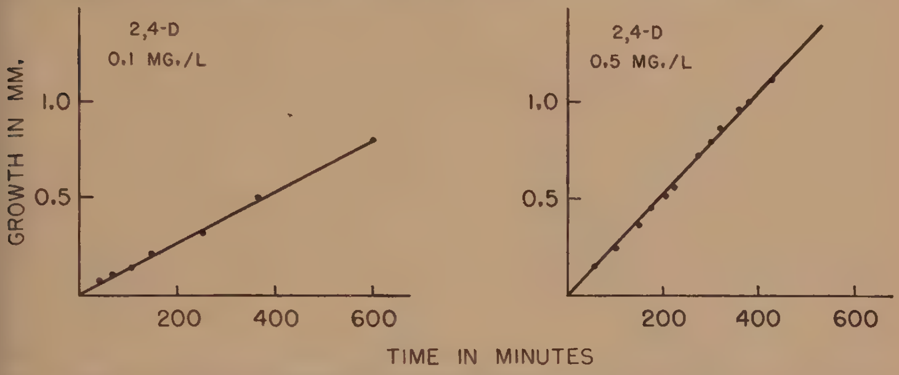

FIG. 5. Progress curves for growth of coleoptile sections over short time intervals. The auxin used in these experiments is 2:4-D.

It may be further noted that Bennet-Clark and Kefford, by choosing to use coleoptiles which are younger and shorter (1·5–2 cm.) than those employed by us (2·75–3·25 cm.), have thereby selected conditions which increase the absolute growth rate of the control (no added auxin) sections and decrease the incremental response to added auxin. A direct comparison of the growth responses obtained by Bennet-Clark and Kefford with those obtained by ourselves is given in Fig. 6. The fact that residual growth of control sections increases with decreasing size of the coleoptiles from which the sections are cut has been pointed out by several investigators, including Bentley (1950).

DISCUSSION

The kinetic treatment of auxin-induced growth proposed by Foster, McRae, and Bonner (1952) has been questioned by Bennet-Clark and Kefford

(1954) on the basis that the growth rate of *Avena* coleoptile sections in response to auxin is not constant with time and does not represent a true initial reaction velocity. Bennet-Clark and Kefford have not, however, used the experimental conditions previously shown to be suitable for the obtaining of constant *Avena* coleoptile section growth rates. They have, rather, selected different conditions which are unsuited to this purpose. The use of short plants decreases the difference in growth rate between sections with and without added auxin. The use of unneutralized auxin solutions and the absence of buffer permits the higher auxin concentrations to cause irreversible damage to the sections merely by acidity. The absence of pH control permits of variation of the pH of the nutrient solution with time and it has long been known that the growth rate of excised coleoptile sections is highly pH-dependent (Bonner, 1934, 1936). The omission of potassium ions from the medium not only depresses growth rate in response to auxin (Thimann and Schneider, 1938), but also decreases the length of time over which auxin-induced growth continues (Thimann and Schneider, 1938; Cooil, 1952). Bennet-Clark and Kefford have suggested that the presence of added ions may obscure a rapid initial response of coleoptile sections to auxin. It may be noted, however, that in our experiments as well as in those of Thimann and Schneider (1938) the effect of potassium ions in maintaining growth rate is marked after the first few hours.

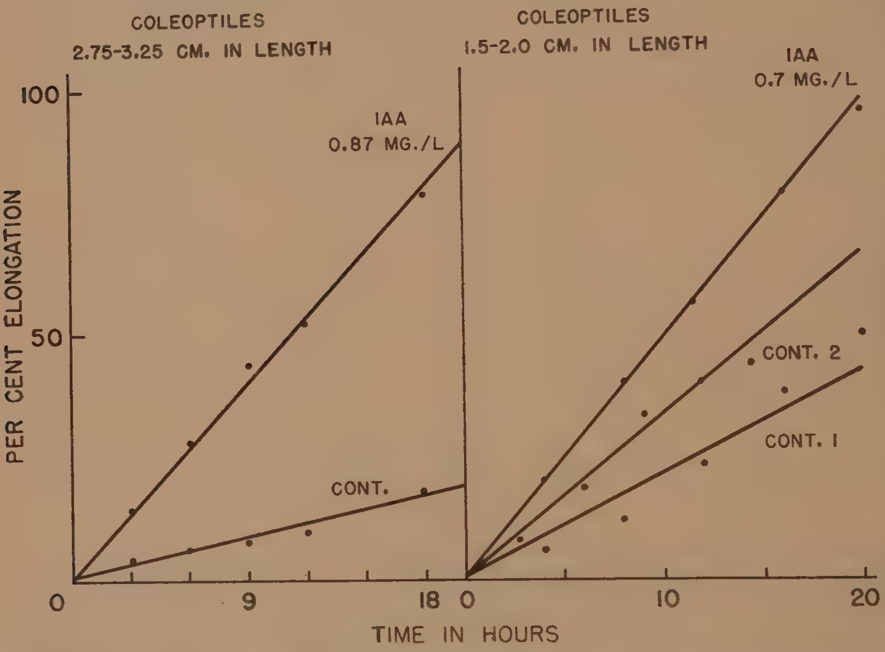

FIG. 6. Comparison of elongation of coleoptile sections with or without added IAA and under two sets of experimental conditions. Left: Data from this paper. Right: Data from Bennet-Clark and Kefford (1954).

The direct comparison of growth rates of *Avena* coleoptile sections in the presence of a high auxin concentration and in the presence or absence of added potassium ions and buffer (Fig. 3) also does not support the suggestion of Bennet-Clark and Kefford.

It is clear, then, that the basic criticism of our work by Bennet-Clark and Kefford, namely that the growth rate of *Avena* coleoptile sections is not constant over the first 12 hours, is unjustified. It is now of interest to examine the data of Bennet-Clark and Kefford in more detail and to consider to what extent their results actually differ from our own. Their data for the higher auxin concentrations need not be further considered since as shown above their results are due to lack of pH control and lack of K ions in their nutrient medium. The data of Bennet-Clark and Kefford for low auxin concentrations and for growth periods up to 20 hours are reproduced in Fig. 7. In each case the data fit a straight line through the origin and in no case does a point deviate from such a line by more than twice the standard error of the measurement as given by Bennet-Clark and Kefford. The same is essentially true for the first 12 hours of the control (no added auxin) data, although this may be quite fortuitous. The control growth rates in the two experiments of Bennet-Clark and Kefford differ by approximately 50 per cent. They are evidently not reproducible, owing no doubt to uncontrolled experimental circumstances such as have been noted earlier in this paper. In any case, the data of Bennet-Clark

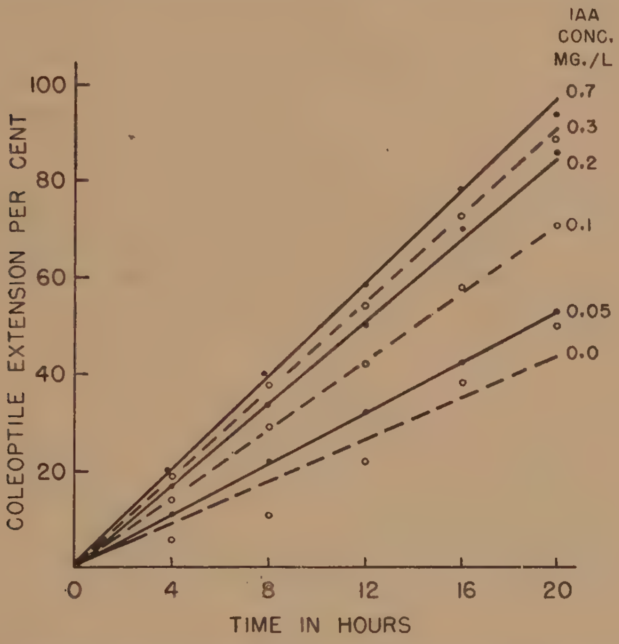

FIG. 7. Progress curve for growth of coleoptile sections at low concentrations of IAA. Data from Bennet-Clark and Kefford (1954).

and Kefford support the notion that growth rate over the initial 12-hour period does not alter greatly with time.

In an earlier paper (Foster, McRae, and Bonner, 1952) we have applied kinetic treatment to the growth inhibition induced by supra-optimal auxin concentrations. We have sought to show that auxin-induced growth inhibition is a necessary consequence of the concept developed elsewhere and by other means that auxin molecules are active in causing growth only after consummation of a two-point attachment with some entity within the plant. Bennet-Clark and Kefford misquote our earlier paper and reverse our position when they say that we hold the view that the data 'demand a two-point attachment'. They also misquote both the word and spirit of our paper in asserting that we claim for ourselves 'a new kinetic treatment based on a two point attachment hypothesis'. The background and development of the kinetics of substrate inhibition is referred by Foster, McRae, and Bonner (1952) to a second paper, that of Foster and Niemann (1953) which deals in detail with the subject. Foster and Niemann point out that kinetic treatment of the inhibition of enzyme action by substrate has been dealt with not only by Lineweaver and Burk (1934), as noted by Bennet-Clark and Kefford, but also and earlier by J. B. S. Haldane in his classic monograph *Enzymes*.

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An Isotopic Study of the Fixation of Nitrogen associated with Nodulated Plants of *Alnus*, *Myrica*, and *Hippophaë*

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SUMMARY

Nodulated plants of *Alnus glutinosa*, *Myrica gale*, and *Hippophaë rhamnoides*, the root systems of which had been exposed to excess free ^{15}N, showed substantial enrichment in fixed ^{15}N content in all parts of the plant, but particularly in the root nodules. The data resemble closely those obtained by the present and previous authors with legumes under comparable conditions, and support the conclusion already drawn from experiments of traditional type that the nodules of these non-legumes are similar to those of legumes in their function and relation to the rest of the plant. Nodulated plants of *Alnus* and *Myrica* continued to fix free nitrogen concurrently with the uptake of combined nitrogen when the latter was supplied in the rooting medium in amounts unlikely to be exceeded in the field. Isotopic tests on detached nodules of one of the species (*Alnus glutinosa*) showed that fixation continued, and though much reduced as compared with that shown by attached nodules it considerably exceeded that experienced by the present and previous authors with detached legume nodules, on the basis of fixation per unit of total nodule nitrogen. It is probable that detached *Alnus* nodules present convenient material for the further study of various aspects of the fixation process.

INTRODUCTION

SUBSTANTIAL evidence of the possession of nitrogen-fixing properties by the root nodules of the genera mentioned in the title has been obtained already by traditional methods (Bond, Fletcher, and Ferguson, 1954). In the work here to be described the more sensitive isotope technique has been employed with the objects of (1) confirming that fixation occurs and that the nodule is the site of the fixation, (2) investigating the effect of combined nitrogen on the fixation, and (3) testing the ability of detached nodules to continue to fix nitrogen.

METHODS

The plants of *Alnus glutinosa* (L.) Gaertn., *Myrica gale* L., and *Hippophaë rhamnoides* L. employed had been grown from seed in the greenhouse in non-aseptic water culture, with Crone's solution (nitrogen-free formula, pH adjusted to a favourable level) as the nutrient solution. The general cultural methods have been described previously (Bond, 1951; Ferguson and Bond,

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1953). The plants were used in the first or second year of growth. In preparation for root enclosure experiments the plants were inserted singly at an early stage in growth into the larger hole of two-hole rubber stoppers which were fitted temporarily to large test-tubes containing culture solution, the plants being then allowed to grow on to the desired stage. The plant was supported in the hole by a split rubber ring.

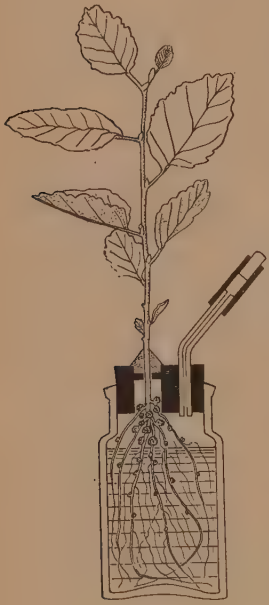

FIG. 1. Arrangement for the enclosure of the root system to allow of its exposure to excess ^{15}N. ($\times \frac{1}{3}$.)

For the actual isotopic test the stoppers bearing the plants were fitted to wide-necked bottles of capacity varying from 50 ml. to 220 ml., depending on the size of the plants. The bottles contained culture solution, a gas space of 15 ml. to 30 ml. (adjusted according to the size of the plant) being left at the top, with the result that the older, larger nodules lay in the gas phase, the rest being submerged. The base of the stem of the plant was sealed to the stopper with plasticine (Fig. 1).

The bottles were attached to a capillary manifold, the whole system evacuated to 0·5 atmosphere, and then gaseous nitrogen containing 36 atom per cent. ^{15}N (prepared as described by Bond and Scott, 1955) admitted until the pressure was restored to 1 atmosphere. The root systems were thus exposed to a gas mixture consisting initially of 10 per cent. oxygen and 90 per cent. nitrogen, the latter containing 20 atom per cent. ^{15}N. Subsequently the plant containers were detached from the manifold and left sealed up in the greenhouse for periods to be indicated later, the mean temperature being approximately 21° C.

In tests with detached nodules, samples of the latter were enclosed together with 1 ml. of culture solution in specimen tubes of 14-ml. capacity, each closed by a rubber stopper with a capillary connexion. The gas phase within the tubes was adjusted to the composition indicated above, by the same procedure, the sealed tubes being then placed in the greenhouse under the conditions of temperature already specified. The average interval between detachment of the nodules and exposure to the isotopic nitrogen was about 30 minutes.

On the expiry of the desired period of exposure to the isotope the plant material was prepared for and submitted to mass spectrometric analysis for ^{15}N content in the manner previously described (Bond and Scott, loc. cit.; also see Acknowledgements to the present paper). Control samples from similar plant material not exposed to the isotopic nitrogen were included in order to provide a check on the analytical procedure. Thirty-five such samples were examined during the investigation, the mean of the results (not all of which are included in the tables) being 0·369 atom per cent., with a standard deviation of 0·014. The mean figure agrees closely with previously published values (Burris, Eppling, Wahlin, and Wilson, 1943). It may be concluded that in the present series of experiments a mass spectrometer value exceeding 0·397 atom per cent. indicates enrichment.

In connexion with the root-system enclosure experiments, the extent to which the excess of free ^{15}N initially provided in the environment of the root system persisted was investigated in the case of an *Alnus* plant set up in the usual way except that the apparatus was slightly modified to facilitate the periodic withdrawal of samples from the gas space in the bottle enclosing the root system. A gas sample taken 2 hours after charging showed a ^{15}N content of 16·4 atom per cent., already lower than the calculated initial value of 20 atom per cent. A sample withdrawn 24 hours after charging proved to have a ^{15}N content of 7·2 atom per cent., while after a further 24 hours the value had fallen to 2·9 atom per cent. This disappearance of ^{15}N is presumed to be due primarily to a process of equilibration whereby the isotope diffuses into the tissues (and perhaps eventually into the external atmosphere via the shoot) and is replaced by ^{14}N coming from the tissues. Nevertheless it will be clear from the results to be presented below that the persistence of the excess of ^{15}N was of sufficient duration for substantial amounts to be fixed. The main disadvantage of the downward drift in ^{15}N concentration is that it introduces an element of uncertainty into the calculation of the total fixation (^{14}N and ^{15}N) during the period of an experiment.

DATA OBTAINED

In initial tests the over-all ^{15}N content was determined of *Alnus* and *Myrica* plants of which the root systems had been exposed to excess ^{15}N (Table I). Although the tests were carried out late in the growing season, significant increases in ^{15}N content over the normal value are shown in all cases, indicative of the occurrence of fixation.

TABLE I

Tests for fixation by nodulated plants of Alnus and Myrica

The *Alnus* plants were 3 months old, with a mean shoot height of 8 cm., the period of exposure to excess ^{15}N being 96 hours. The *Myrica* plants were 4 months old, with a mean shoot height of 4·5 cm., the period of exposure being 120 hours. All tests carried out in late September.

Plant number	^{15}N content of plant as a whole, atom %	
	Alnus	*Myrica*
Root systems exposed to excess ^{15}N		
1	0·508	0·626
2	1·330	0·535
3	0·482	0·409
4	—	0·416
Controls		
5	0·362	0·367
6	0·362	0·368
7	0·364	—

Table II presents data obtained in further tests, carried out earlier in the growing season, in which different parts of the plants were separated prior to analysis. Very substantial enrichment is shown by the nodule tissues, and in all cases their ^{15}N content exceeds that shown by the other parts of the plant. In a corresponding test with *Hippophaë* in which plants 12 cm. in height were used, the mean ^{15}N content of the nodules was 0·586, of the roots 0·540, and of the shoots 0·578 atom per cent.

TABLE II

Distribution of fixed ^{15}N in plants of Alnus and Myrica after exposure of the nodulated root systems to excess free ^{15}N

Alnus plants Nos. 1–3 were 12 months old, with a mean shoot height of 19 cm., and were exposed to excess ^{15}N for 75 hours (in June). *Alnus* plants Nos. 4–6 were 4 months old, with a mean shoot height of 10 cm., the period of exposure being 120 hours (in August). *Myrica* plants Nos. 1–3 were 12 months old, with a mean shoot height of 19 cm., the period of exposure being 116 hours (in July). Nos. 4–6 (combined for analysis) were 4 months old, with a mean shoot height of 10 cm., the period of exposure being 96 hours (in August).

Plant number	^{15}N content, atom %		
	Nodules	Roots	Shoot
		Alnus	
Exposed to excess ^{15}N			
1	0·636	0·608	0·526
2	1·994	0·427	0·455
3	1·734	0·447	0·596
4	1·360	0·731	
5	1·360	1·192	
6	0·898	0·656	
Control			
7	0·371	0·371	0·374
		Myrica	
Exposed to excess ^{15}N			
1	0·662	0·573	0·442
2	0·540	0·394	0·474
3	0·770	0·577	0·513
4, 5, & 6	1·470	0·800	0·578
Control			
7	0·373	0·368	0·370

For purposes of comparison a similar test was carried out with a typical legume, namely red clover (*Trifolium pratense* L.), 3-months-old plants grown in water culture being employed. After 96 hours' exposure (in August) of the root systems to isotopic nitrogen the following ^{15}N contents were shown (mean values for three plants): nodules 0·698, roots 0·462, shoots 0·475 atom per cent. These results are similar to those reported by Burris, Eppling, Wahlin, and Wilson (1942) for other legumes. It is clear that the results presented above for the non-legumes resemble closely those yielded by legumes.

No increase in ^{15}N content was shown by an *Alnus* plant from which the nodules had been removed immediately prior to exposure of the root system to the isotope, the actual values obtained being 0·378 atom per cent. for the roots and 0·369 for the shoot. In addition to indicating that the nodule is the nitrogen-fixing organ, this result also shows that the free ^{15}N supplied to the plants in the present series of experiments was not accompanied by any significant traces of gaseous compounds (such as ammonia or oxides of nitrogen) containing ^{15}N and assimilable by plants. Had any such contaminant been present, then the ^{15}N content of the plant would have been expected to show enrichment. Further evidence on this score was obtained in tests with four non-nodulated plants of *Alnus* previously supplied with combined nitrogen but transferred to nitrogen-free solution prior to exposure of their roots to ^{15}N. Subsequent analyses of these plants showed ^{15}N contents (over-all) of 0·360, 0·377, 0·370, and 0·375 atom per cent. respectively, i.e. no enrichment had occurred.

In an experiment on the effect of the presence of combined nitrogen on the functioning of the nodules of *Alnus*, plants were employed which had been supplied with 50 mg. of ammonium-nitrogen per litre of culture solution throughout their development, a level which, as tests with non-nodulated plants show, is sufficient to sustain vigorous growth without nodule nitrogen. The same level of combined nitrogen was present at the commencement of the isotopic tests, and suitable measurements showed that each plant absorbed approximately 2·3 mg. of this combined nitrogen during the 3 days of the test. The ^{15}N data (Table III) show that a substantial fixation of free nitrogen accompanied the use of combined nitrogen. In a corresponding test with *Myrica* a still higher level of ammonium-nitrogen (140 mg. per litre) was supplied in the culture solution, the over-all ^{15}N contents of the four plants proving to be 0·412, 0·407, 0·377, and 0·412 atom per cent. respectively after exposure to the isotope. Thus fixation was associated with three of the plants.

TABLE III

Test for fixation of free nitrogen by nodulated Alnus plants supplied with combined nitrogen

The plants were 3½ months old, the mean shoot height being 22 cm. The culture solution contained ammonium sulphate in amount providing 50 mg. ammonium-nitrogen per litre of culture solution. The root systems were exposed to excess ^{15}N for 72 hours (in July).

Plant number	^{15}N content, atom %		
	Nodules	Roots	Shoot
Exposed to excess ^{15}N			
1	0·902	0·398	0·475
2	0·902	0·430	0·458
3	0·850	0·429	0·505
Controls			
4	0·377	0·376	0·356
5	0·371	—	0·366

The last results to be presented refer to tests for the continuation of fixation in nodules after detachment from the plant. Data for eight samples of *Alnus* nodules exposed to excess ^{15}N are provided in Table IV. It is clear that fixation occurred in all the samples. The highest fixation was shown by nodules from first-year plants tested in August (samples 5 and 6). The results of a comparable test with detached soya-bean nodules are also included in the table, one only of the samples showing evidence of fixation, and that relatively slight.

Table IV

Tests for fixation by detached Alnus nodules, with comparable results for soya-bean nodules

Each sample was of 1 g. (*Alnus*) or 2 g. (soya-bean) fresh weight, the samples being exposed to excess ^{15}N for 48 hours. The tests on *Alnus* samples 1–4 were carried out in October, those on samples 5–8 in August, samples 5 and 6 being taken from first-year plants, 7 and 8 from second-year plants. The tests with soya-bean nodules were performed in July, the nodules being taken from water-culture plants at an early flowering stage, inoculated with Wisconsin Strain 505 of the nodule organism.

Sample number	^{15}N content, atom %	
	Alnus	Soya bean
Exposed to excess ^{15}N		
1	0·419	0·398
2	0·408	0·380
3	0·407	0·382
4	0·405	0·395
5	0·476	—
6	0·490	—
7	0·451	—
8	0·456	—
Controls		
9	0·366	0·375
10	0·370	0·368
11	0·369	—
12	0·367	—

DISCUSSION

The data presented in Tables I and II in respect of *Alnus* and *Myrica*, and given in the text for *Hippophaë*, confirm that fixation of nitrogen is associated with nodulated plants of these genera. Calculation from the data in Table II of the actual increases in ^{15}N content over the mean control figure of 0·369 atom per cent. shows that in *Alnus* (plants 1–3) the mean increase for nodule samples is 1·086 atom per cent., for roots 0·125, and for the shoot 0·157. The corresponding figures for *Myrica* (plants 1–6) are 0·492, 0·217, and 0·133 atom per cent. The disparity between the nodules and the rest of the plant would no doubt have been still greater had it been possible to maintain the initial high concentration of free ^{15}N in the environment of the root system (p. 305). The especial accumulation of the newly fixed ^{15}N in the nodules can only be explained satisfactorily on the basis that the nodule is the site of the fixation, with subsequent translocation of a proportion of the products of fixation to other parts of the plant. Supporting evidence is provided by the demonstration that the removal of the nodules results in the cessation of fixation by the plant, while again plants grown without nodules do not fix nitrogen (p. 307). The parallel with the results obtained in isotopic tests with legumes, as instanced by the data for red clover, is obviously a very close one, and the conclusion previously reached (Bond, Fletcher, and Ferguson, 1954) that in their function and relation to the rest of the plant the nodules of these non-legumes resemble closely those of legumes now receives further confirmation.

These findings apply in the first instance to the nodules of plants cultivated in the greenhouse, but it is very unlikely that such nodules differ radically in their activities from those normally present on plants in the field; certainly in their cytology and general characteristics the greenhouse nodules resemble closely the field type. Direct evidence of fixation by field nodules is, however, now being sought.

The question of the identity of the agent conferring or inducing the faculty of fixation in these nodules must be approached with caution. It is well known that there is uncertainty as to the systematic position of the endophytes of non-legume root nodules; some investigators of the cytology of the nodules have taken the view that more than one organism is regularly present. The resolution of these uncertainties may have to await the development of techniques whereby nodules can be reconstituted by the use of pure cultures of the endophytes, rather than by that of suspensions of crushed nodules, the method employed in the present work.

It had already been shown (Bond, Fletcher, and Ferguson, 1954) that in short-term experiments with *Alnus* and *Myrica* the presence of combined nitrogen in the culture solution leads to increased absolute development of nodules, although the weight of nodules formed relative to that of the rest of the plant is reduced. The question whether these nodules are active in the fixation of free nitrogen is conveniently studied by the ^{15}N technique. Table III shows that *Alnus* nodules were in fact quite active in fixation despite the presence of a fairly high level of combined nitrogen in the culture solution, and exhibited as great an accumulation of ^{15}N on the atom per cent. basis as the nodules of some plants in nitrogen-free solution (Table II). The difficulty in calculating total fixation has already been indicated (p. 305), but if it is assumed that the mean proportion of free ^{15}N in the environment of the root system over the period of the experiment to which Table III refers was 5 atom per cent., then, since the total nitrogen contents of the various parts of the plants are known, calculations can be made which indicate that the mean fixation per plant of ^{14}N and ^{15}N together amounted to 0·8 mg., as compared with the 2·3 mg. of combined nitrogen taken up by the plant during the same period. Three out of four *Myrica* plants supplied with a high level of combined nitrogen continued to show some utilization of free nitrogen (p. 307), though probably less than with plants in nitrogen-free solution (Table I).

The chief reason for examining the effect of combined nitrogen on fixation was in relation to the question (to which reference has already been made) of how active in fixation these non-legume nodules are under field conditions. One approach is to test with greenhouse plants the effect on nodule function of factors likely to be operative in the field. It is concluded that levels of combined nitrogen unlikely to be exceeded in the field do not suppress fixation in *Alnus* and *Myrica*. There is not the same opportunity for or interest in this type of experiment with *Hippophaë*, since nodulation is severely reduced in the presence of combined nitrogen (Bond, Fletcher, and Ferguson, 1954).

Of the nodules of the three non-legume genera concerned, those of *Alnus* only have so far been tested for the continuation of fixation after detachment from the plant. As noted already, consistently positive results were obtained, the accumulation of fixed ^{15}N on the atom per cent. basis being considerably greater than that shown in comparable tests with detached nodules of a legume (soya-bean). The author's experience with the latter is in agreement with that of Tove, Niss, and Wilson (1950), who obtained only occasional positive results in isotopic tests with nodules of soya-bean and cow pea. Only by modifying the procedure so that mass spectrometric analyses are carried out not on the whole of the nodule nitrogen but on an acid extract containing chiefly the metabolically active fraction has it proved possible to obtain consistent and indubitable evidence of fixation in detached legume nodules. Using this procedure Aprison and Burris (1952) showed that in soya-bean nodules exposed to 20 atom per cent. ^{15}N the isotope content of the nitrogen of the acid extract rose to a maximum of 0·57 atom per cent. after 6 hours. Magee and Burris (1954) extended the demonstration to other legume genera.

The position is thus that fixation in detached *Alnus* nodules is on a sufficient scale to raise the ^{15}N content of the nodule nitrogen as a whole by a significant amount, whereas this is not consistently the case in legume nodules. It seems likely that detached *Alnus* nodules will provide favourable material for the further study of the non-legume fixation process, and perhaps of symbiotic fixation in general.

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Oxidative Activity of Particles prepared from Lettuce Seedlings

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SUMMARY

A particulate fraction similar to mitochondria was isolated from germinating lettuce seedlings.

The endogenous oxygen uptake of this fraction was very high. It could oxidize most of the Krebs cycle intermediates, the oxidation of *l*-malate being particularly marked.

Glutamic, glycollic, and formic acids could also be oxidized to a certain extent.

Fatty acids inhibited the endogenous respiration, the inhibition increasing with increased concentration. Coumarin and thiouren do not seem to have any direct effect on the respiratory enzyme systems. They apparently affect the respiration of whole seeds only indirectly, maybe through some germination regulating mechanism not yet known.

THE effect of light, temperature, and coumarin on germination of lettuce seeds was reviewed by Evenari (1952). Audus and Quastel (1947) observed the dual effect of coumarin as germination inhibitor and growth inhibitor; they made a suggestion that it forms easily dissociating compounds with some enzymes or metabolites. More recently Evenari *et al.* (1954), Leopold and Guernsey (1954), and Levari (1954) showed that rather complicated interrelations exist between the intensity of respiration and the treatment of seeds with light of various qualities or with chemicals like 2:4-D and coumarin. It was interesting therefore to find out whether this is a direct effect of the treatment on the respiratory enzymes or the net result of a series of processes occurring in the germinating seed, the equilibrium of which is somehow upset by the treatment.

As not very much is known about the respiratory system of lettuce seeds, the presence of the various enzymes had to be established. Particulate fractions were isolated from lettuce seedlings germinated in water, and their oxidative abilities were investigated. Also the effects of coumarin as germination inhibitor and of thiourea as having the reverse action were studied.

MATERIAL AND METHODS

Lettuce seeds of a light-insensitive variety 'Progress' were used throughout the experiments.[2] One-gramme lots of sterilized seeds were germinated in Petri dishes with 8 ml. of water or of the solution to be tested; they were

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[2] The seeds were a gift from Ferry Morse Seed Co., California.

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germinated in a constant temperature room at 26° C. for various lengths of time. At desired intervals, lots from five Petri dishes were harvested and a particulate fraction was isolated as described by Hackett and Simon (1954). The particles were washed three times and finally suspended in 6 ml. of suspending medium. Measurements of oxidative activity were carried out at 30° C. All the materials were placed directly into the main compartment of the Warburg flask, the total volume in the flask being 1·5 ml. Nitrogen determinations were done on parallel samples using the Nesslerization method.

EXPERIMENTAL

Utilization of different substrates. The preparations possessed a very high endogenous oxygen uptake, which increased with prolongation of germination period from approximately 30 μl. O_2/mg. N/hour after 24 hours' germination to over 200 μl. O_2/mg. N/hour after 96 hours' germination. This endogenous respiration could not be eliminated even with as many as five washings. It was not due to contamination and could therefore be attributed to the presence of either an intraparticulate or some water-insoluble substrate. In all the experiments 'no substrate' controls were run to provide the correction for endogenous respiration.

Particulate preparations from seedlings of various ages were supplied with succinate and α-ketoglutarate as substrates and the oxygen uptake of the system was measured. The results are given in Table I. As shown in the table, the maximal activity is found in particles from 72-hour-old seedlings. But even these activities are much lower than those reported for mitochondria prepared from other plants (Millerd, 1951; Dow, 1952; Davies, 1953; Laties, 1953; Hackett and Simon, 1954).

TABLE I

Oxidative activity of particles prepared from lettuce seedlings of various ages

Reaction mixture contains: sucrose, 0·2 M.; phosphate, 5×10^{-2} M.; $MgSO_4 \cdot 7H_2O$, 2×10^{-3} M.; ATP, 10^{-3} M.; $MnCl_2$, $0{\cdot}5 \times 10^{-3}$ M.; substrate, 2×10^{-2} M. Particulate suspension, 0·5 ml. The results are given as oxygen uptake in μl. O_2/mg. N/hour and are corrected by subtraction of the endogenous respiration.

Age of seedlings	Succinate	Ketoglutarate
Dry seeds	<10	<5
24 hours	60	60
48 hours	55	40
72 hours	160	130
96 hours	140	120

Other acids from the tricarboxylic acid cycle were used as substrates with the lettuce particles; the results are given in Table II. A few interesting points emerge from these results. The preparations are capable to some extent of oxidizing acetate; this ability is increased by addition of pyruvate. Glutamate is also oxidized by the system, which may indicate the presence of some amino-acid oxidase. Addition of malate increases the oxygen uptake more than any other acid. Citrate, it seems, is not oxidized at all; Plaut and Plaut (1952) have reported a similar case of inability to oxidize citrate by guinea-pig heart-muscle mitochondria and a similar explanation may also be applied here, although it has to be remembered that citric acid is also the least accumulated acid in lettuce leaves (Schneider, 1941). Malic is the acid responsible for more than 57 per cent. of the total acidity in lettuce leaves (Schneider, 1941). The concentration of malate giving optimal oxygen uptake with the particulate preparations is as low as 2×10^{-3} M. (see Fig. 1). The particles are able to a certain extent to oxidize also glycolate and formate (Table III), so that a possibility exists of some other cycle operating instead of or in addition to the ordinary tricarboxylic acid one.

TABLE II

Oxidation of various substrates by particles prepared from lettuce seedlings

Reaction mixture as in Table I. Results given as $\mu l.\ O_2$/mg. N/hour corrected for endogenous respiration, and as percentage increase on endogenous respiration.

Substrate	Substrate conc. in M.	Oxygen uptake as $\mu l.\ O_2$/mg. N/hour	Increase in oxygen uptake as percentage of endogenous
Pyruvate	2×10^{-2}	20	10
Citrate	2×10^{-2}	none	—
α-Ketoglutarate	2×10^{-2}	130	70
Succinate	2×10^{-2}	160	80
l-Malate	10^{-2}	210	115
l-Malate	10^{-5}	10	—
l-Malate + Pyruvate	$10^{-5} + 2 \times 10^{-2}$	30	15
Acetate	2×10^{-2}	30	15
Acetate + Pyruvate	$2 \times 10^{-2} + 2 \times 10^{-2}$	80	45
Acetate + Glutamate	$2 \times 10^{-2} + \mu$M/flask	90	50
Glutamate	1μM/flask	70	40

TABLE III

Relative increase in oxygen uptake on addition of various substrates

Reaction mixture as in Table I. Substrate concentration 2×10^{-2} M.

Endogenous	100
Glycolate	139
Formate	146

Lettuce seeds are fatty seeds; it was thought therefore that the high endogenous oxygen uptake may be due to some water-insoluble fatty acids which may be there as products of fat breakdown. Fatty acid oxidases may be present in the preparations. Fatty acids were tried as substrates. As is evident from Table IV, they either did not affect the oxygen uptake or inhibited the endogenous respiration. The inhibition decreased with decreased concentration (see Fig. 2). Also preliminary experiments show that addition of cytochrome diminishes the inhibitory effect. Therefore the question of the presence of fatty acid oxidases in the particles remains undecided.

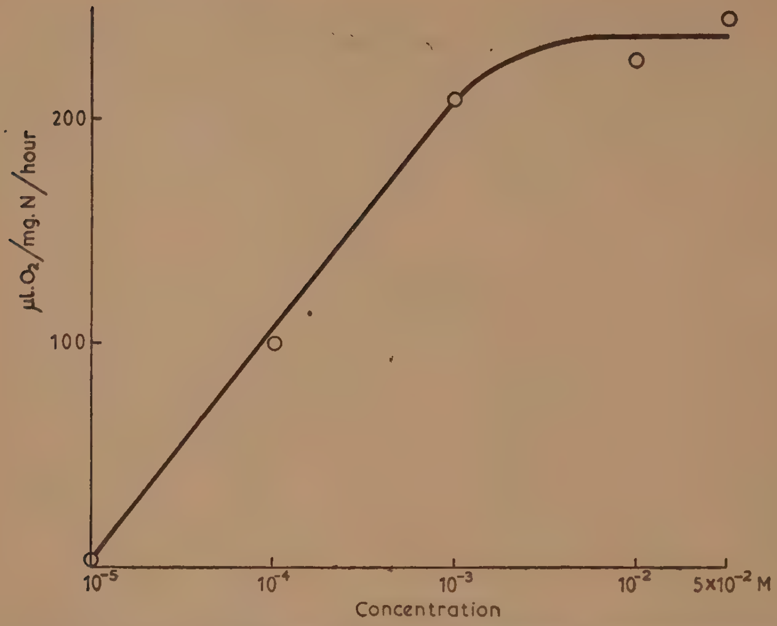

FIG. 1. Effect of substrate concentration on the rate of oxidation by particles prepared from lettuce seedlings. Substrate: *l*-Malate. Otherwise reaction mixture as in Table I. Figures are corrected by endogenous oxygen uptake, and represent the first-hour measurements. Age of the seedlings, 72 hours.

TABLE IV

Relative oxidation of fatty acids by lettuce particles

Reaction mixture as in Table I. Substrate concentration 2×10^{-2} M. as potassium salt.

Endogenous . . .	100
Lauric (C_{12}) . . .	50
Capric (C_{10}) . . .	40
Caprylic (C_8) . . .	85
Caproic (C_6) . . .	106
Butyric (C_4) . . .	102
Oleic	106

Effect of coumarin and thiourea. The effect of coumarin and thiourea on the activity of the particulate preparations was studied. Addition of thiourea to the reaction mixture, to the concentration of 125 mg. per cent., inhibited the oxygen uptake by 40 per cent. when α-ketoglutarate was the substrate. Addition of coumarin to 20 mg. per cent. in otherwise the same conditions increased the oxygen uptake by about 20 per cent.

When seeds were germinated in coumarin and thiourea respectively, of concentrations stated above, the particulate fractions isolated from them had activities similar to the water controls. The endogenous respiration was not

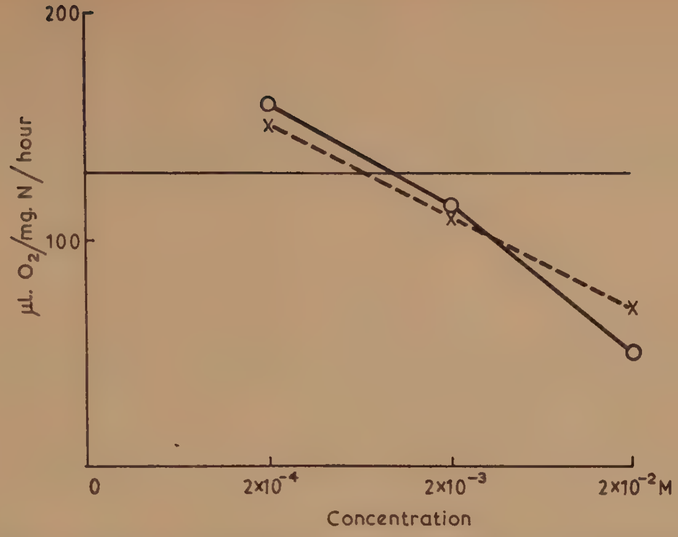

FIG. 2. Effect of substrate concentration on rate of oxidation by particles prepared from lettuce seedlings. Endogenous oxygen uptake (————); capric acid (O——O——O); lauric acid (x---x---x). Reaction mixture as in Table I. The acids used as K-salts. Age of seedlings, 72 hours.

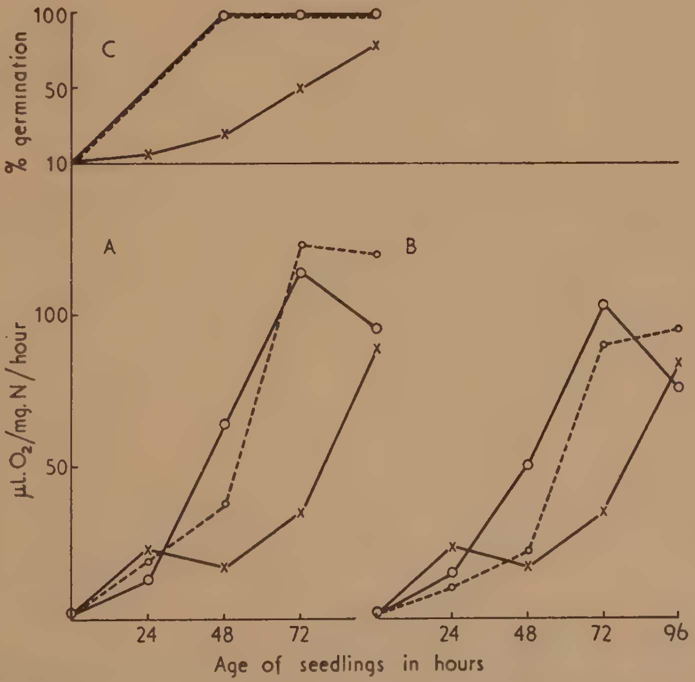

FIG. 3. Effect of treatment on oxidation rate by particles prepared from lettuce seedlings of various ages. Seeds germinated in water (O——O——O); seeds germinated in thiourea (125 mg. %) (O----O----O); seeds germinated in coumarin (20 mg. %) (x——x——x). Reaction mixture as in Table I. A, Substrate succinate 2×10^{-2} M.; B, Substrate α-ketoglutarate 2×10^{-2} M.; C, Germination percentage of the seeds used for particles preparation. Oxygen uptake values corrected by endogenous oxygen uptake.

affected by thiourea, but coumarin delayed germination and also delayed the increase in endogenous respiration and substrate oxidation. Fig. 3 shows the curves of oxygen uptake of particles prepared from seedlings of various ages germinated in water, coumarin, and thiourea and also shows the changes in germination percentage.

DISCUSSION

As no information was available about the respiratory pathways of lettuce seedlings, it is interesting to know that the necessary oxidative systems for the oxidation of acids of the tricarboxylic acid cycle are present in the isolated particles. But the presence alone of these enzymatic systems does not prove that this cycle is either the only or the main route of oxidation. The main substrate after the first 24 hours is the storage fat (Poljakoff-Mayber, 1952). This is also indicated by the lower R.Q.s of this variety (Levari, 1954). It could be calculated from Levari's results that the amount of oxygen taken up by the seeds in the first 24 hours exceeds the necessary amount for oxidation of the sugar that has disappeared during that period.

When trying to identify the substrates present in the germinating seeds by paper chromatography, no volatile fatty acids of the C_2–C_7 were found (Poljakoff-Mayber and Mayer, 1955). Lactic acid was present in relatively large quantity and small amounts of malic acid were found. The other acids of the Krebs cycle could not be detected because of large amounts of interfering substances not yet certainly identified.[1] There is therefore no certain evidence for or against the Krebs cycle as a predominant pathway for substrate oxidation in the germinating lettuce seedling. The marked oxidation of malic acid and the possibility to oxidize glycolic and formic acids may in a way support Barron's idea (1951) of an alternative route for malate oxidation. Supporting evidence for that was already supplied by Link *et al.* (1952).

From the data of Table I it is evident that enzyme activity per mg. nitrogen increases with prolongation of germination time in a manner similar to the course of change in proteinase activity of lettuce seeds (Poljakoff-Mayber, 1953).

Coumarin delayed the germination of the seeds and also delayed the increase in particulate activity (Fig. 3), so that the oxygen uptake per mg. nitrogen in the preparation is directly proportional to the percentage of germination (Fig. 4). As coumarin does not affect directly any of the respiratory enzymes, the delayed particle activity or decrease of respiration of whole seeds (Levari, 1954) is an indirect effect.

Thiourea inhibits the rate of oxidation *in vitro*, delays a little the rise in oxidative activity *in vivo* (Fig. 3), but it does not seem that its dormancy-breaking properties are in any way related to any of these effects.

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[1] The identification of the organic acids was done for us by Dr. L. E. Bentley, Bedford College, London.

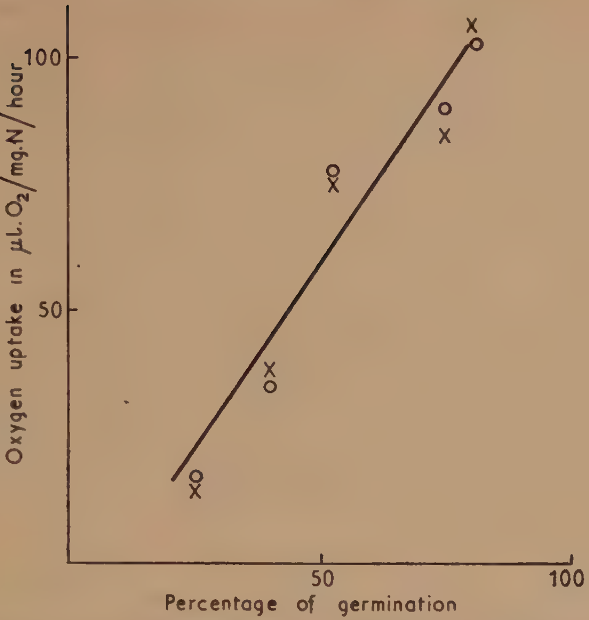

FIG. 4. The relation between oxidative activity and germination percentage of particles prepared from lettuce seedlings germinated in coumarin (20 mg. %). Succinate as a substrate O; α-ketoglutarate as a substrate ×. Values corrected for endogenous oxygen uptake.

ACKNOWLEDGEMENTS

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The Effect of IAA on the Oxidative Activity of Mung-bean Mitochondria

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SUMMARY

The effect of IAA on the oxidative activity of mitochondria was tested with a variety of substrates. Addition of IAA did not increase the oxygen uptake of the system in any of the cases. Toxic concentrations of IAA caused decreases in oxygen uptake.

IAA, within a certain limit of concentrations, was found to reverse the depressant effect of alcohol on the endogenous oxygen uptake.

MANY varied views have developed during the last two decades concerning the nature and the mechanism of action of the plant growth substances. Numerous workers have shown the close interrelations between elongation growth, as a net result of the hormone effect, and increases in respiration. Observations showing growth inhibition by using respiratory inhibitors supported the idea that the plant growth hormones act through metabolic pathways, preferably with some interrelation with respiratory enzymes (Bonner, 1933; Thimann, 1949; Bonner and Wildman, 1947).

The necessity of malate, or some other acid pretreatment, for demonstrating the increase in respiration during auxin-induced growth of *Avena* coleoptiles suggested to Thimann (1949) that the SH-enzyme necessary for growth is almost certain to be one of the dehydrogenases.

Audus and Garrard (1953), on the other hand, working with pea-root segments, came to the conclusion that there is no evidence for any direct effect of auxin on the respiratory enzymes of growing roots.

It was interesting, therefore, to study this problem by using isolated multi-enzyme systems in the form of particulate preparations and testing the effect of IAA on their oxidase systems.

Mung-bean seeds were germinated and grown as described by Millerd (1951); the particulate fractions were isolated as described by Hackett and Simon (1954). Oxygen uptake was measured in ordinary Warburg manometers at a temperature of 30° C. The results are given as μl. O_2/mg. N/hour and are corrected by subtraction of the endogenous respiration which is very low. Nitrogen was estimated by the Nesslerization method on separate samples.

The *in vitro* effect of IAA on the oxidizing activity of the particles was tested by its addition to the concentration of 5 mg./l., directly into the reaction

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mixture. Various acids were used as substrates. The results are summarized in Table I. Although the means of the IAA-containing samples are usually a little lower than the controls, the difference is very small and not significant. The conclusion may be drawn that this concentration of the hormone has no

TABLE I

The in vitro *effect of IAA on the oxidative activity of Mung-bean mitochondria*

Reaction mixture: inorganic phosphate, 0·05 M.; sucrose, 0·2 M.; $MgSO_4 \cdot 7H_2O$, 2×10^{-3} M.; $MnCl_2$, $0{\cdot}5\times10^{-3}$ M.; ATP, 10^{-3} M.; substrate is stated below. Flasks with IAA contain 5 mg./l. IAA. Results as μl. O_2/mg. N/hour.

	Mean	S.D.	S.E.
Succinate 2×10^{-2} M. alone	374	16·9	6·9
,, ,, +IAA	364	28·9	11·7
Citrate 2×10^{-2} M. alone	221	10·0	4·5
,, ,, +IAA	214	10·0	4·0
Malate 2×10^{-3} M. alone	107	9·9	4·9
,, ,, +IAA	104	9·3	4·6
Malate 2×10^{-3}+pyruvate 2×10^{-2} M. alone	142	6·5	3·2
,, ,, ,, ,, +IAA	142	3·4	1·7
Pyruvate 2×10^{-2} M.	42	—	—
No substrate without IAA	40	5·2	2·6
,, +IAA	38	10·3	5·1

direct effect on the activity of the tricarboxylic acid cycle oxidases. But as the concentration used is already nearly supra-optimal for growth, the resulting small decrease in oxygen uptake might be due to the toxic effect of auxin. To test this assumption, toxic concentrations of IAA (70 mg./l.) were added to the grinding medium, thus prolonging the contact between the IAA and the enzymes. This high concentration was also maintained in the reaction mixture. The results are summarized in Table II. They show a marked decrease in oxygen uptake by the IAA-treated particles, the mean difference being significant at a 2 per cent. level.

Leopold and Guernsey (1953) suggested the formation of an energy-rich IAA-Co A compound, apparently showing the optimal concentration of IAA for its formation to be of the order of 10^{-8} M. Formation of such a compound should also affect the oxidases and interfere with their activity. Therefore concentrations of this order were used. Results of oxygen uptake measurements are summarized in Table III. The three separate experiments in that table show a rather high degree of variability, but the calculated means suggest no effect of IAA on the oxidases in the particles.

Thimann's (1949) pretreatment idea was also tested. *l*-Malate was added into the grinding medium to a concentration of 10^{-2} M. so that the particles were pretreated with the acid in the cold for 3 hours before the addition of the IAA, which was added directly into the Warburg flask. The results are given in Table IV, and show that the oxygen uptake of malate-pretreated particles is inhibited markedly by addition of IAA, the difference being significant at between 5 and 2·5 per cent. level.

TABLE II

The in vitro *effect of IAA on citrate oxidation by Mung-bean mitochondria*

Reaction mixture as in Table I. Citrate concentration 2×10^{-2} M. IAA incorporated into grinding medium to concentration of 70 mg./l. Controls ground without IAA. Final concentration of IAA in IAA-treated flasks is also 70 mg./l. Results as µl. O_2/mg. N/hour.

No. of experiment	Control	IAA-treated
Expt. No. 1	167	116
	169	118
	159	99
	126	104
Expt. No. 2	124	112
	106	116
	194	102
	161	118
Expt. No. 3	155	143
	153	142
	—	163
	—	161
Mean	151	125
S.D.	25·8	22·5
S.E.	8·3	6·6

TABLE III

The in vitro *effect of IAA on citrate oxidation by Mung-bean mitochondria*

Reaction mixture as in Table I. Citrate concentration 2×10^{-2} M. Concentration of IAA in IAA-containing flasks 10^{-8} M. Results as µl. O_2/Mg. N/hour. Difference non-significant.

No. of experiment	Control	IAA
Expt. No. 1	191	147
	190	163
Expt. No. 2	186	171
	146	166
Expt. No. 3	144	165
	150	167
Mean	168	163
S.D.	23·3	8·4
S.E.	9·6	3·5

When oxidative phosphorylation was measured, the fluctuations of the P/O ratio in the different experiments were rather high, and there was not much difference between the IAA-treated and untreated particles, the variations in the ratios being in the controls between 0·3 and 1·3 and in those of the IAA-treated material between 0·3 and 1·0, with citrate as a substrate.

It may be concluded, therefore, that IAA in its physiological concentration has no direct effect on the activity of the tricarboxylic acid cycle oxidases. It does not seem likely that it exerts any marked influence on the high energy

TABLE IV

Effect of malate pretreatment and addition of IAA on oxidative activity of Mung-bean mitochondria

Reaction mixture as in Table I. IAA concentration when present 5 mg./l. *l*-Malate incorporated in the grinding medium to concentrations of 10^{-2} M. Final *l*-Malate concentration $3{\cdot}7 \times 10^{-3}$ M. Results as μl. O_2/Mg.N/hour.

No. of experiment	*l*-Malate	*l*-Malate+IAA
Expt. No. 1	94·0 93·0	77·5 76·5
Expt. No. 2	91·5 80·5	78·5 71·0
Expt. No. 3	78·5 73·5	63·5 77·0
Mean	85	74·0
S.D.	8·7	5·8
S.E.	3·6	2·7

phosphate bond formation either, though the toxic effect of IAA may be to some extent attributed to interference with the normal oxidative processes.

The possibility of some indirect effect of IAA on the activity of the particles was considered. The auxin, in the living cell, could affect the formation of the enzymes or their physical organization on the particles. Therefore, mitochondrial preparations were isolated from Mung-bean stems grown in the ordinary way in parallel with others which were grown for the last 24 hours with their roots in IAA solution (5 mg./l.). The results are given in Table V. There is a marked decrease in oxygen uptake by particles isolated from IAA-treated plants. But as the auxin was fed to the roots and the concentration nsed is toxic for roots, it occurred to us that this effect may be due to a secondary influence, as a result, for example, of some deficient water absorption through the damaged roots.

TABLE V

In vivo *effect of IAA on citrate oxidation by Mung-bean mitochondria*

Reaction mixture as in Table I. Citrate concentration 2×10^{-2} M. No IAA in reaction mixture. Results as μl. O_2/Mg. N/hour.

	Control	IAA-treated
	258·0	205·5
	255·5	239·0
	264·0	236·0
	223·5	208·0
	207·0	191·5
	251·0	212·0
	231·0	206·0
	215·5	174·5
	233·0	222·5
Mean	237·6	209·8
S.D.	20·3	20·7
S.E.	6·7	6·9

Another experiment was designed where Mung-bean hypocotyls were soaked in parallel in water and IAA solution (5 mg./l.) for 24 hours and then the particulate preparations were made from the two lots. The oxygen uptake of such preparations with citrate as a substrate is given in Table VI. These results support the idea that the IAA effect on growth is totally independent of the tricarboxylic acid cycle enzymes.

TABLE VI

In vivo *effect of IAA on citrate oxidation by Mung-bean mitochondria*

Reaction mixture as in Table I. Citrate concentration 2×10^{-2} M. No IAA in reaction mixture. Results in μl. O_2/Mg. N/hour. Difference non-significant.

No. of experiment	Control	IAA-treated
Expt. No. 1	285 243	245 272
Expt. No. 2	247 238 262	243 208 —
Mean	255	272
S.D.	73	75

In general these results confirm the results of Dow (1952), who also could not find any specific effect of growth hormones on the respiratory enzymes.

As a result of an accidental treatment of a particulate preparation with an alcoholic solution of IAA, a rather surprising and at present quite unexplained mutual interaction of alcohol and IAA came to light.

The situation briefly is that within a fairly narrow concentration range (see Table VII) alcohol markedly depresses the normal endogenous respiration of

TABLE VII

Effect of various concentrations of alcohol and IAA on oxygen uptake by unwashed mitochondria

Concentrations of IAA and alcohol as stated below, otherwise reaction mixture as in Table I.

Content of the flask	μl. O_2/Mg. N/hour		
	Expt. 1	Expt. 2	Expt. 3
IAA acid 10^{-2} M. Alcohol 0·1 ml./flask . . .	20	47	42
No IAA. Alcohol 0·1 ml./flask	10	26	20
IAA potass. salt 10^{-2} M. Alcohol 0·1 ml./flask . .	33	37	25
No IAA. Alcohol 0·1 ml./flask	20	20	10
IAA acid 10^{-5} M. Alcohol 0·1 ml./flask . . .	10	12	—
No IAA. Alcohol 0·1 ml./flask	10	14	—
IAA potass. salt 10^{-2} M. Alcohol 0·01 ml./flask . .	39	25	64
No IAA. Alcohol 0·01 ml./flask	31	29	50
IAA potass. salt 10^{-2} M. Alcohol 0·001 ml./flask . .	37	—	—
No IAA. Alcohol 0·001 ml./flask	37	—	—

the particles. Addition of IAA to such depressed mitochondria roughly doubles the rate of oxygen uptake per hour. The same is true for mitochondria prepared from Alaska peas (see Fig. 1). The mechanism of the depressant action of alcohol and the reversal by IAA have not been examined and are merely reported as a further rather minor 'effect caused by IAA'.

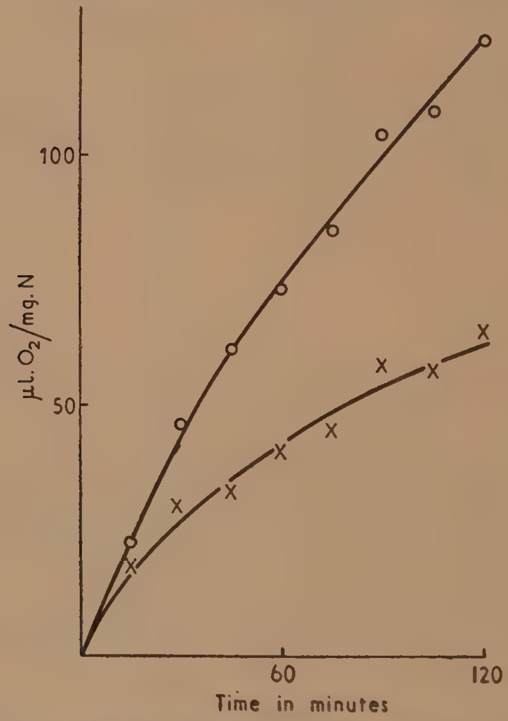

FIG. 1. Effect of IAA and alcohol on oxygen uptake by unwashed mitochondria of Alaska peas. Experiments run for 2 hours. Alcohol 0·01 ml./flask. IAA concentration 10^{-2} M. Otherwise reaction mixture as in Table I. The values in the graph are means of four experiments. × Alcohol only; ○ Alcohol+IAA

DISCUSSION

The lack of influence of IAA on mitochondrial oxidative activity with a variety of treatments and pretreatments which have been employed suggests strongly that the point of action of IAA as a promoter of increased growth is not via the respiratory enzyme systems. That there is increased respiration of whole growing tissues following treatment with IAA is undoubted, but it seems clear that this increase in respiration is caused by the increased growth or cell extension producing a renewed supply, for example of phosphate acceptors.

The present work does not suggest that IAA has any direct effect either on synthesis of respiratory enzymes or on their activity in multi-enzyme systems.

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The Interactions of Auxins and Anti-auxins in the Stimulation of Root Growth

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SUMMARY

Experiments on the interactions of auxins and anti-auxins in roots have been extended to studies with concentrations giving growth stimulations using 2-mm. sections excised from the extension zone of roots of *Pisum sativum*. The curves relating growth responses to $\log_{10}$ concentration for β-indolylacetic acid (IAA) and three anti-auxins, α-(1-naphthylmethylsulphide) propionic acid (NMSP), 1-naphthylmethylsulphide acetic acid (NMSA), and 4-chloro-3-nitrobenzoic acid (CNB), are of very similar shape. A fourth anti-auxin, *p*-chlorophenoxy-*iso*-butyric acid (PCIB), shows negligible stimulation of growth in any concentration. In multifactorial experiments involving stimulatory concentrations of IAA (10^{-4} and 10^{-5} p.p.m.) with several stimulatory concentrations of the first three anti-auxins (NMSP, NMSA, and CNB), consistent mutual antagonisms were clearly demonstrated. PCIB in non-inhibitory concentrations markedly antagonized stimulation by IAA. Similar mutual antagonisms were shown in various mixtures of two anti-auxins.

Both the similarities of the concentration-response curves and the consistent mutual antagonisms suggest that both auxins and anti-auxins in stimulating root growth are exerting identical physiological actions in the same growth system. On the assumption that these substances are active when adsorbed at some enzyme or other protoplasmic surface, it has been demonstrated that these experimental results are more easily explained by a direct action at those growth centres than by a competitive antagonism of a natural endogenous growth inhibitor, as suggested in a previous paper.

The bearing of these results on current theories relating auxin and anti-auxin activities to molecular structure is discussed.

INTRODUCTION

FOR a very long time now it has been held that natural auxin (i.e. β-indolylacetic acid, IAA) is present in the elongating cells of roots in supra-optimal concentrations, such that its augmentation by an external application of hormone gives the well-known inhibition of extension growth. Upon this assumption the classical auxin theory of geotropic responses of roots was founded. It has been given added support in recent years by the discovery that certain homologues of auxins, which antagonize auxin action in its inhibition of root growth, may considerably promote the extension growth of roots when applied by themselves in low concentrations (Åberg, 1950, 1952; Burström, 1950). The antagonist would cause this by displacing the effective internal concentration of the endogenous auxin towards its optimum value. Such a

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stimulation of root growth has been accepted by some workers as one of the primary criteria for recognizing an 'anti-auxin'.

This would constitute a very simple and rational approach to the study of auxins and anti-auxins and facilitate investigations into the relationships of their activities to molecular structure, were it not for the fact that IAA itself may, in very low concentrations, cause a small, though quite well established, stimulation of extension growth. Such a state of affairs has led the writer to suggest (Audus and Shipton, 1952) that both auxins and anti-auxins may have virtually the same action on root growth when supplied externally. Stimulation by both types of compound may be brought about by an identical antagonism of a natural growth inhibitor present in the extending cells. The degree of stimulation by the compound (auxin or anti-auxin) would be determined by the extent to which normal growth is being suppressed by this inhibitor and the effectiveness of the applied compound as its antagonist. In addition to such antagonistic actions, both compounds, at much higher concentrations, would themselves be direct inhibitors, presumably at different growth centres. This would be necessary to explain the well-known inhibiting effect of auxins and anti-auxins in high concentrations.

The antagonism of auxin inhibition by anti-auxins means that these two classes of compound must compete with each other for the growth centres where this inhibition is taking place. The anti-auxin, having a very low inhibiting activity, may replace the highly active auxins at these centres, thus partially relieving the auxin inhibition.

Since auxins and anti-auxins show this clear-cut interaction in the inhibition of root growth, it is to be expected that they will also show interactions in the stimulation of root growth in low concentrations. So far all the experimental data have come only from studies at high *inhibiting* concentrations and these, as has been previously indicated (Audus and Shipton, 1952), may throw little light on the nature of auxin and anti-auxin *stimulation* of root growth.

The present paper describes the results of a series of multifactorial experiments on the extension growth of root sections to determine the extent and nature of the interactions at low stimulating concentrations, using the auxin β-indolylacetic acid (IAA) and four anti-auxins, α-(1-naphthylmethylsulphide) propionic acid (NMSP) (Åberg, 1950), 1-naphthylmethylsulphide acetic acid (NMSA) (Åberg, 1951), *p*-chlorophenoxy-*iso*-butyric acid (PCIB) (Burström, 1950), and 4-chloro-3-nitrobenzoic acid (CNB) (Minarik *et al.*, 1951).

MATERIAL AND METHODS

The plant material for this study was 2-mm. sections excised from the extending zone of the radicles of 2-day-old seedlings of *Pisum sativum* (var. 'Meteor', Messrs. Sutton & Sons, Reading). Samples of 10 sections were grown in complete darkness at 25° C. in 10 ml. of 0·5 per cent. sucrose solution and the extension followed at intervals for 48 hours. All seedlings were germinated in sterile sand in complete darkness at 25° C. in a saturated atmosphere. The method of excising the sections has already been described in detail (Audus and Shipton, 1952; Audus and Garrard, 1953). During the growth period the Petri dishes containing the growing sections were rocked slowly on an electrically driven device. Such agitation has previously been shown to be necessary to obtain optimal growth (Audus and Garrard, 1953). Absolute sterility of the sections was not possible, but the growth of bacteria and fungi was kept down to negligible proportions in these experiments by using sterilized solutions, glass-ware, and instruments.

At suitable intervals during the growth period Petri dishes were removed from the agitator and the section extension measured. Two methods were employed. In one, sections were removed from the culture fluid, rapidly dried on sterile filter paper, and weighed to the nearest 0·05 mg. on a micro-torsion balance. They were then returned to the culture medium. In the other, measurements of length to the nearest 0·05 mm. were made with a travelling microscope without removal from the dish. Both give a direct measure of extension growth, since sections do not show any change in thickness during extension except in high auxin concentrations. The first method has the advantage of speed and was used in the majority of experiments. Length measurements were used where appreciable growth inhibitions were expected. The Petri dishes were then returned to the agitator. Average growth rates were thereby measured over four periods, 0–4 hours, 4–7 hours, 7–24 hours, and 24–48 hours from excision.

In the interaction experiment two concentrations of IAA were studied in combination with a range of concentrations of each anti-auxin. These two concentrations, 10^{-5} p.p.m. and 10^{-4} p.p.m., are those that have been shown to give maximum growth stimulation in pea sections. The anti-auxins were usually used in a wider range of concentrations. The multifactorial experiments were planned on a strictly orthogonal basis so that full statistical analyses of variance could be carried out on them. The number of samples of sections that can be conveniently handled in one experiment is strictly limited and so each major interaction experiment was broken down into a series of smaller experiments in which the two IAA concentrations were studied with one anti-auxin concentration at a time. Each unit experiment was usually performed three times. In view of the small effects expected with many of the treatments, a considerable degree of replication was provided within unit experiments to ensure a high degree of precision and unequivocal demonstration of small interactions. The results of all such unit experiments were pooled and the analysis of variance carried out on the whole data.

The three compounds NMSP, NMSA, and PCIB were chosen because of their high stimulatory activities reported in the literature. CNB was chosen largely because of its easy synthesis in quantity. Both NMSP and NMSA, at concentrations at which they themselves have very little effect on growth of roots, have been shown by Åberg (1950, 1951) to reduce considerably the inhibition of flax root growth by a number of auxins, including IAA. A similar antagonism of IAA and 2:4-dichlorophenoxyacetic acid (2:4-D) inhibition of pea-root section growth has been clearly demonstrated in this laboratory in the unpublished experiments of M. E. Brownbridge. PCIB has been shown by Burström (1950) to accelerate greatly the growth in length of wheat roots, but the apparent antagonism of IAA action by this compound in his experiments might be explained as an additive effect of the two compounds. Extensive experiments with pea-root sections by one of us (L. J. A.) have demonstrated, however, that a definite antagonism of IAA inhibition (at a concentration of 0·01 p.p.m.) can be demonstrated over a very narrow range of PCIB concentrations just on the threshold of its own inhibition (4–8 p.p.m.). At slightly higher concentrations PCIB is highly toxic. CNB has been shown to antagonize markedly 2:4-D inhibition of root growth (Minarik *et al.*, 1951).

The bulk of the NMSP used in the experiments was provided by Pal Chemicals Limited of London. Other small samples essential for the completion of the work and also the sample of NMSA were obtained from Professor Årne Fredga of the University of Uppsala and Dr. Börge Åberg of the Royal Agricultural College, Uppsala. The sample of PCIB was provided by Professor R. L. Wain of Wye College, University of London. The CNB was prepared by one of us (L. J. A.) by nitration of *p*-chlorobenzoic acid using a standard technique. The product was crystallized three times from dilute ethanol and had a sharp melting-point at 183·5° C.

The effects of growth substances acting alone

Concentration–growth response curves were determined for each compound acting alone. It was found that the nature of this response curve varied markedly with the particular phase of section growth considered. With IAA the maximum response, to both stimulatory and inhibitory concentrations, was obtained in the first few hours. Thence the response declined until, after 24 hours, very little effect of any concentration, stimulatory or inhibitory, could be detected. These results are shown in Fig. 1. It seems possible that the disappearance of response is due to a rapid inactivation of the IAA by root tissue and perhaps by contaminating micro-organisms, but these possibilities have still to be checked experimentally. Another explanation, along the lines of the two-phase cell-extension theory of Burström (1942), might be advanced. The above limitation of the stimulating action of IAA to the first phase of section growth would conform well to his theory but unfortunately, in the above experiments, the inhibiting action of the higher concentrations is similarly confined to the first phase, and not, as Burström's theory suggests, to the second phase.

The behaviour of NMSP differs from IAA in that usually no response was obtained in the first 4 hours and maximum response was not observed until the 7–24-hour period (see Fig. 3). The response subsequently declined. The most likely explanation of this delayed response is that NMSP penetration into the growing cell is a slow process and maximum equilibrium concentrations of this compound are not reached until 6–7 hours after immersion in the culture fluid. On the other hand, the acetic acid homologue NMSA evokes an immediate and maximal response in the first 4 hours, which means a rapid rate of entry for this compound. It is difficult to see why two such closely similar molecules should enter the cell at such greatly different speeds and it may be that the delayed action of NMSP has some other physiological explanation. These well-founded differences, however, serve to stress the

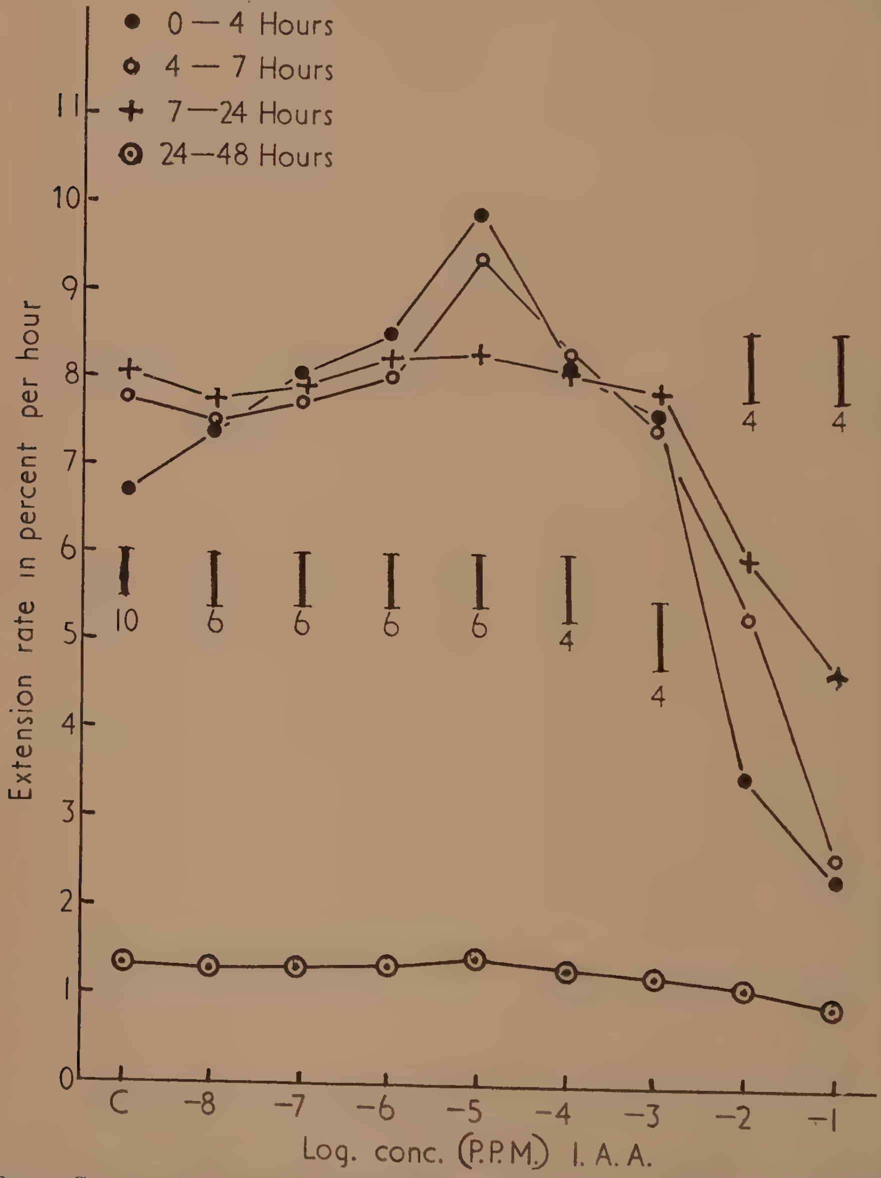

FIG. 1. Concentration–response curves for the action of β-indolylacetic acid (IAA) on the growth of excised pea-root sections showing the changes in response taking place during the course of the extension. The vertical lines represent the least significant differences at the 5% probability level between growth-rates at the relevant concentrations. The figures below the lines record the numbers of replicate samples from which each mean was calculated.

caution that must be exercised in the interpretation of concentration–growth response curves for sections, particularly if only a single long growth period is employed (Audus, 1952; Bennet-Clark and Kefford, 1954).

With CNB an immediate response is obtained, and this persists for 24 hours

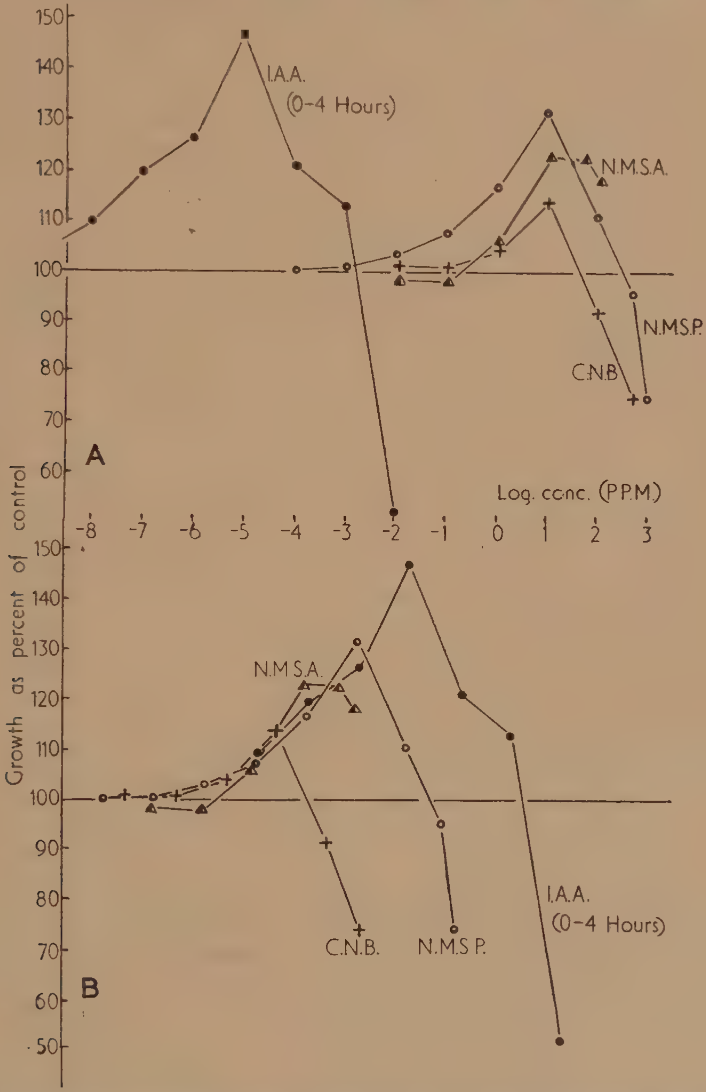

FIG. 2. Concentration–response curves for the action of β-indolylacetic acid (IAA) and three anti-auxins (NMSP = α-(1-naphthylmethylsulphide) propionic acid, NMSA = 1-naphthylmethylsulphide acetic acid and CNB = 4-chloro-3-nitrobenzoic acid) on the growth of excised pea-root sections.

A. Plotted strictly against log. concentration in parts per million. B. Curves shifted without further alteration along the concentration axis until their suboptimal rising portions coincided. The anti-auxin curves are from over-all extensions in the first 24 hours. The IAA curve is for the maximal growth response of the first 4 hours.

but tends to disappear in the last phases of growth (see Figs. 7 and 8). Presumably entry of this compound is as rapid as that of IAA, but the persistence of the response throughout the whole growth period suggests that it is not destroyed in the root tissues.

PCIB, in contrast to its marked stimulation of the growth of intact roots obtained by Burström (1950), gives no marked stimulation of section growth in any of the concentrations used (Fig. 6).

However, in spite of these differences in behaviour in relation to the phase of extension growth, the broad nature of the *optimum* growth responses is remarkably similar for these compounds (Fig. 2A). The shapes of the ascending limbs of the stimulation curves for IAA, NMSP, NMSA, and CNB are very alike. This is clearly shown in Fig. 2B, where the four curves of optimum response have been superimposed. It is not difficult to imagine that such stimulation is brought about by precisely the same physiological action of each compound, the only differences being in their 'activity' or 'effectiveness', manifested by a lateral shifting of the whole response curve along the concentration axis. Above the optimum concentration the curve declines rapidly into the inhibitory region of growth response. It is reasonable to suppose that this inhibition is due to a process very largely independent of the stimulation (see Hellström, 1953, and later, p. 342), and that therefore the concentration giving maximum growth is a false optimum, set where the increase in growth in the ascending limb of the stimulation response curve is halted and reversed by the onset of this inhibition. On this basis, therefore, the inhibition region begins at some point slightly below the optimum concentration. This independent 'inhibiting' action in high concentrations sets in differently in the various compounds. With NMSP it first appears at a concentration about 1,000 times higher than that which just produced a stimulation. For NMSA high inhibiting concentrations could not be studied, since only very small amounts of material were available. It seems likely that the difference between the threshold concentrations for stimulation and inhibition for IAA is even greater if one considers the optimum responses of the first 4 hours after excision. For CNB the inhibiting phase appears much earlier so that the maximum stimulation obtained is correspondingly smaller. Over the concentration ranges studied the inhibitions are directly proportional to the logarithm of the concentration and the slopes of these curves are very similar for the four compounds.

The joint actions of anti-auxins and IAA

(*a*) *Interaction with NMSP.* The results of the full experimental series are shown in Fig. 3.

The delayed action of NMSP and the rapid decline in the effect of IAA makes the investigation of direct interaction of effects very difficult. Variance analysis of the full experimental series, however, shows that in the first two growth periods there is no significant interaction between the effects of these two substances, which can be interpreted to mean that the NMSP may not have penetrated in sufficient quantities into the cell to exert an effect on growth or to disturb the effective IAA concentrations at the growth centres. In the 7–24-hour curves, however, the interaction variance is high and very significant. Inspection of the graphs of Fig. 3 shows that this is due to a marked reduction in the stimulatory effects of NMSP by both concentrations of IAA, in spite of the fact that IAA at this time has very little effect on the growth rate.

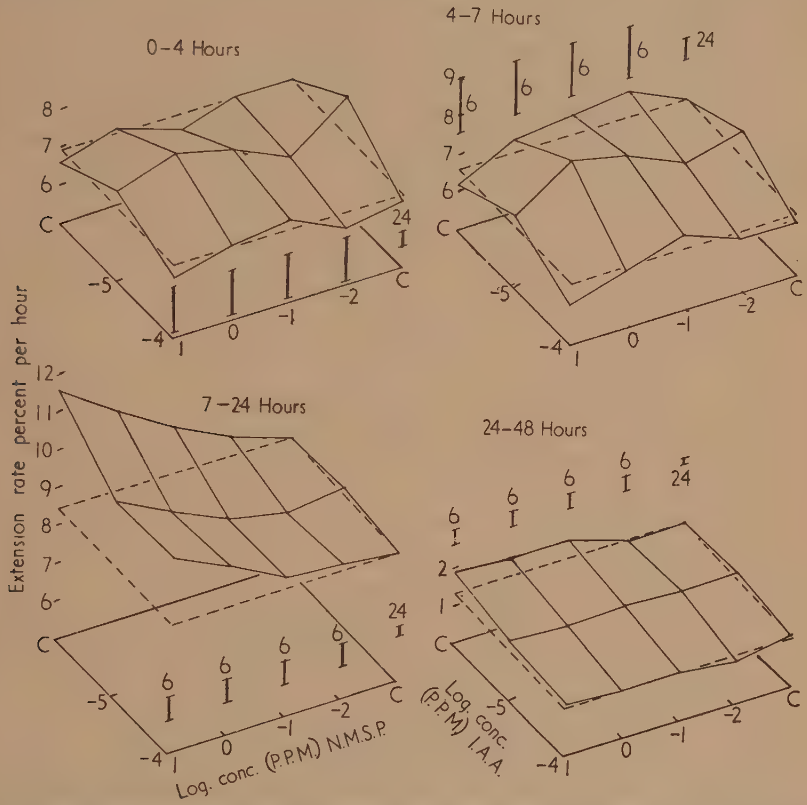

FIG. 3. Three-dimensional graphs showing the interactions between β-indolylacetic acid (IAA) and α-(1-naphthylmethylsulphide) propionic acid (NMSP) in the stimulation of extension growth of excised pea-root sections. The times noted above each graph are the growth periods after excising from the parent root. The vertical lines represent the least significant differences at the 5% probability level between the means of growth-rates in the series of concentration combinations against which they are placed. The figures against these lines record the numbers of replicate samples from which each mean of the series was calculated.

There are four possible interpretations of this latter effect. Firstly, residual IAA may directly antagonize NMSP at the growth centres, although its own stimulating action may have passed off (see theory on p. 344). Secondly, it might be suggested that the IAA, after penetrating into the cell and there evoking the early growth response, is converted into an inactive (or perhaps even an inhibitory) derivative which itself antagonizes NMSP action. This would agree well with the recent suggestions of Bennet-Clark and Kefford (1954), who interpreted their growth rate–time curves for coleoptile sections in high auxin concentrations in terms of inhibitor production from IAA. Thirdly, it is possible that the lower growth rate in IAA in this period may result from the higher growth rate in the first two periods. If optimal total extension is in fact limited, under the action of both types of compound, by other unknown factors, then a stimulation in an early phase will necessarily mean a slower rate of growth in a later phase. This seems unlikely, since the interaction disappears in the final period of 24–48 hours. Although the growth rate at this time is low, the total extension involved is quite appreciable and one would have expected therefore still to find a much lower growth rate in the auxin-treated sections. The fourth possibility is that IAA still present in the external solution may impede the entry of NMSP and thus reduce the degree of its stimulation in this phase.

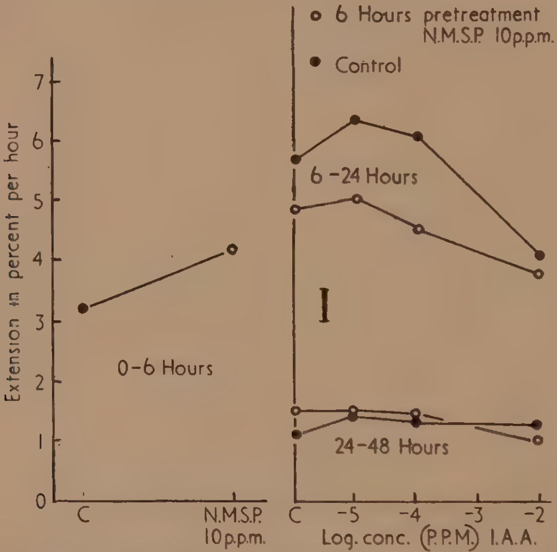

FIG. 4. Graphs showing the effects of 6 hours' pretreatment of root sections with 10 p.p.m. of α-(1-naphthylmethylsulphide) propionic acid (NMSP) followed by washing in 0·5% sucrose solution on the subsequent growth-rate and response to β-indolylacetic acid (IAA). The vertical line shows the least significant difference between means at the 5% probability level.

An attempt to test this last alternative was made by carrying out a slightly modified experiment in which a series of samples were pretreated by growing them for a period of 6 hours after excision in a solution of NMSP in 0·5 per cent. sucrose at a concentration of 10 p.p.m. A similar series in 0·5 per cent. sucrose solution alone served as controls. Penetration of NMSP was confirmed by the slight stimulation of growth rate obtained over this pretreatment period. The samples were subsequently thoroughly washed in 0·5 per cent. sucrose solution and then grown in 0·5 per cent. sucrose solutions containing a range of IAA concentrations, two stimulatory (10^{-5} p.p.m. and 10^{-4} p.p.m.) and one inhibitory (10^{-2} p.p.m.). The results are shown in Fig. 4. Over the

18-hour period subsequent to pretreatment, sections pretreated with sucrose alone gave normal growth responses to IAA. Those samples pretreated with NMSP, however, all showed a much smaller growth than the controls after washing and, in addition, the stimulatory IAA effects were reduced to far below the 5 per cent. level of significance. Inhibition in 10^{-8} IAA was also much smaller than in controls but was still significant.

It is probable, therefore, that the NMSP which had entered the sections antagonized both the stimulatory and inhibitory action of IAA which entered later. This suggests that IAA and NMSP do interact directly at the growth centres and supports the theory that the interactions of the main experiment are due to a direct antagonism of NMSP action either by residual IAA in the cells or by a transformation product of it. This theory is also supported by a single experiment using the rapidly acting NMSA where antagonism of its action by stimulatory concentrations of IAA was demonstrated in the first 4-hour period of growth (Fig. 5). But mutual antagonism of entry cannot yet be entirely ruled out.

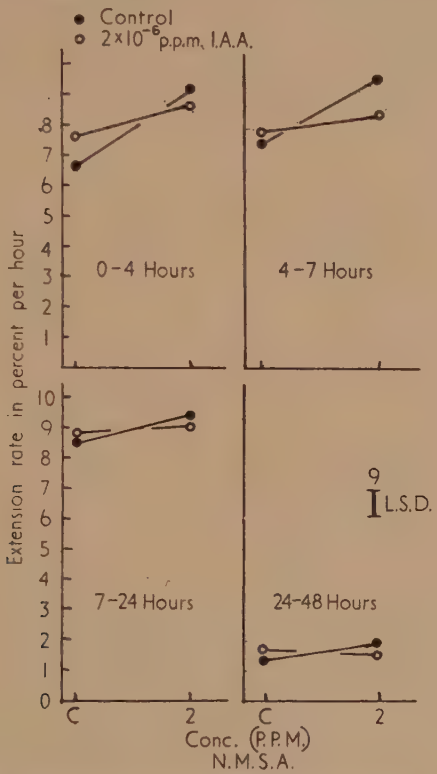

FIG. 5. Graphs showing the interaction of β-indolylacetic acid (IAA) and 1-naphthylmethylsulphide acetic acid (NMSA) and its variation with time in the stimulation of extension growth in excised pea-root sections. The concentrations of both compounds were suboptimal. The vertical line shows the least significant difference between means at the 5% probability level.

The inhibitory effect of NMSP pretreatment on subsequent section growth in its absence in the external medium is much more puzzling. It cannot be interpreted in terms of the limitation of total extension by other unknown factors (see third alternative on p. 336) since (*a*) growth continues at a high rate if NMSP is not removed by washing, and (*b*) the small extra extension occurring during the pretreatment period is very much smaller than the subsequent reduction of extension after washing. This aspect of NMSP action needs further attention.

(*b*) *Interaction with PCIB.* As we have previously noted, PCIB has very little stimulatory effect on the growth of root sections. A very slight stimulation is indicated at a concentration of 3 p.p.m., but this scarcely reached the 5 per cent. level of significance in the majority of our experiments. This small effect was, however, observable in the first few hours of section growth. This compound must therefore enter the sections rapidly. In the interaction experiment shown in Fig. 6 this effect was well below the 5 per cent. significance level. With the two growth-stimulatory concentrations of IAA there is a highly significant interaction in the 0–4-hour growth period ($P < 0{\cdot}1$ per cent.). This interaction consists of a complete abolition of the IAA stimulation in all the PCIB concentrations studied. In the 5–24-hour period this interaction disappears completely as does the IAA effect itself.

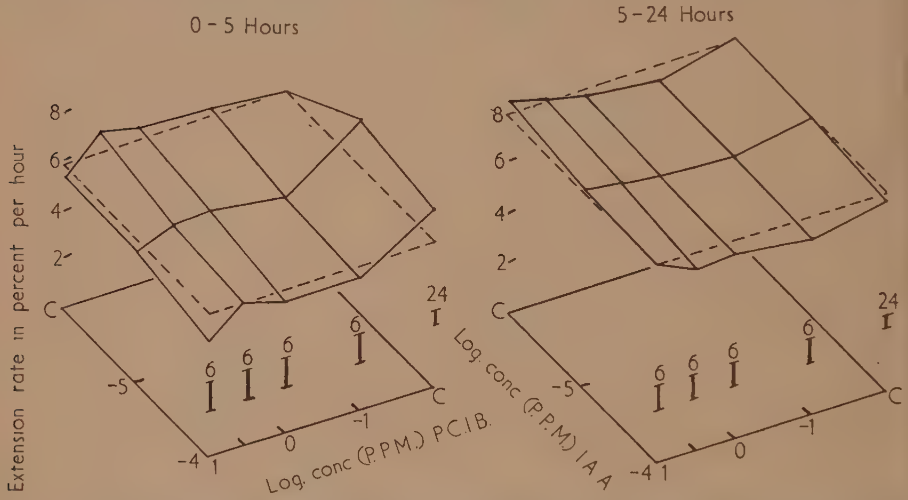

FIG. 6. Three-dimensional graphs showing the interactions between β-indolylacetic acid (IAA) and *p*-chlorophenoxy- *iso*-butyric acid (PCIB) in the stimulation of extension growth of excised pea-root sections. The times noted above each graph are the growth periods after excision from the parent root. The vertical lines represent the least significant differences at the 5% probability level between the means of growth-rates in the series of concentration combinations against which they are placed. The figures against these lines record the numbers of replicate samples from which each mean of the series was calculated.

(*c*) *Interaction with CNB.* The results of this experiment series are shown in Fig. 7. Statistical analysis of the extension data of the complete experiment shows significant interactions in the first three growth phases. From 0–4 hours P is $< 0{\cdot}1$ per cent., from 4–7 hours $< 1{\cdot}0$ per cent., and from 7–24 hours < 5 per cent. This interaction takes the form of a mutual antagonism of stimulation, as Fig. 7 clearly shows. Thus many of the combinations of two stimulatory concentrations gave growth rates which were smaller than those in either of the two concentrations alone. In some combinations (e.g. IAA and CNB at 0·1 p.p.m. from 4–7 hours) growth rates were reduced to the control level. All effects and interactions disappeared after 24 hours.

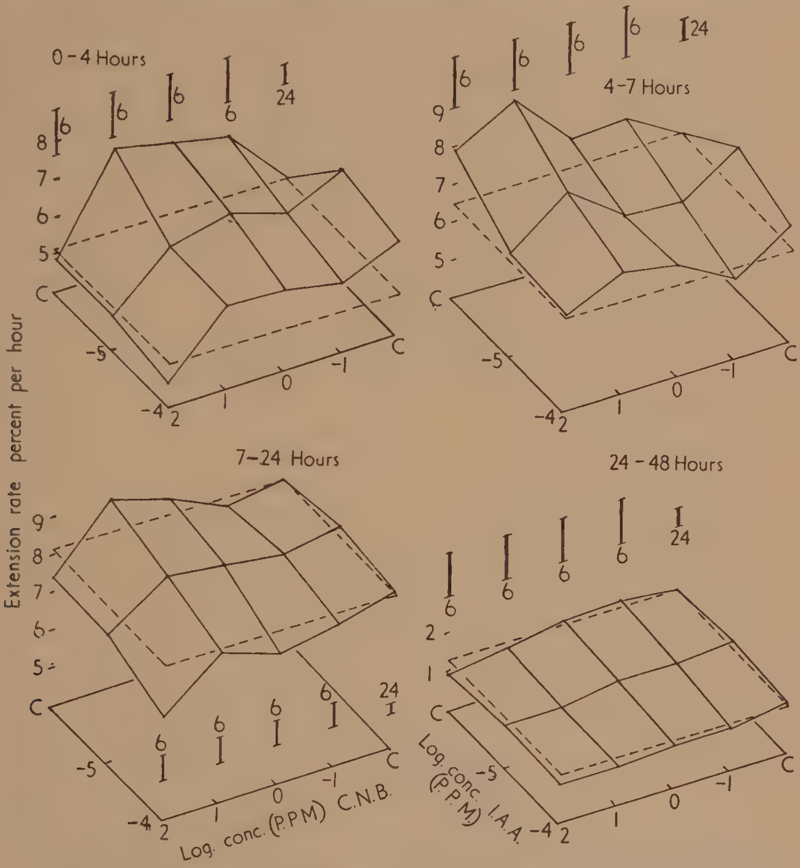

FIG. 7. Three-dimensional graphs showing the interactions between β-indolylacetic acid (IAA) and 4-chloro-3-nitrobenzoic acid (CNB) in the stimulation of extension growth of excised pea-root sections. The times noted above each graph are the growth periods after excision from the parent root. The vertical lines represent the least significant differences at the 5% probability level between the means of growth-rates in the series of concentration combinations against which they are placed. The figures against the lines record the numbers of replicate samples from which each mean of the series was calculated.

These mutual antagonisms between auxin (IAA) and anti-auxins in their stimulatory effects on root section growth further support the hypothesis that their physiological actions are identical. This possibility should naturally be explored further by studying the mutual interactions of the anti-auxins themselves.

The mutual interaction of the anti-auxins

(*a*) *The interaction of NMSA and CNB.* The interaction between the rapidly acting NMSA and CNB is illustrated in Fig. 8, where the results of the full experimental series are drawn. An analysis of variance of this data shows

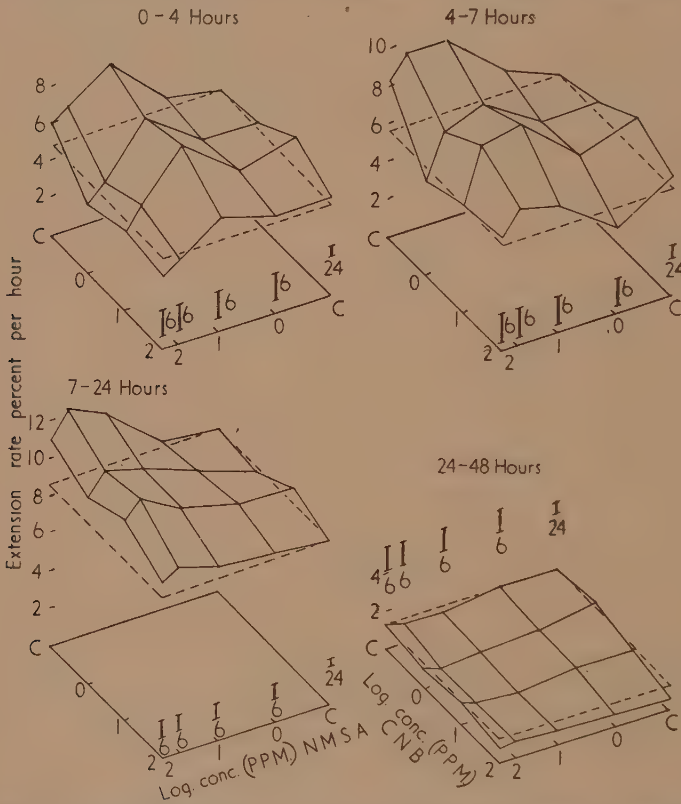

FIG. 8. Three-dimensional graphs showing the interactions between 1-naphthylmethylsulphide acetic acid (NMSA) and 4-chloro-3-nitrobenzoic acid (CNB) in the stimulation of extension growth of excised pea-root sections. The times noted above each graph are the growth periods after excising from the parent root. The vertical lines represent the least significant differences at the 5% probability level between the means of growth rates in the series of concentration combinations against which they are placed. The figures against the lines record the numbers of replicate samples from which each mean of the series was calculated.

these interactions to be highly significant in the first three periods measured, i.e. 0–4, 4–7, and 7–24 hours ($P \ll 0{\cdot}1$ per cent.). Again they take the form of a mutual antagonism of stimulation of precisely the same nature as that of the CNB–IAA interaction.

(*b*) *Other anti-auxins.* It has not yet been possible, owing to difficulties in obtaining materials, to carry out any extensive experiments with other anti-auxins having marked powers to stimulate root growth, but experiments on a rather simpler plan than those above have shown that PCIB at one part per million, at which it has itself no significant effect on the growth rate, definitely antagonizes the stimulatory action of 10 p.p.m. NMSP. (From variance analysis $P = 1$ per cent.)

DISCUSSION

It is clear that both in their individual stimulatory and inhibitory effects on root section extension growth and in their mutual interactions there is *nothing* to distinguish in principle one compound from another in the group of five already tested. PCIB does admittedly differ from the rest in that, in the experiments so far performed, it has a very low and somewhat elusive stimulatory activity, but it is felt that with more extended experiments having a greater degree of precision than those already performed, the effect might well be established as real. The logical conclusion from this correspondence of effects is that, whatever the situation in shoots and coleoptiles and in the *inhibition* of root growth, these compounds are *stimulating* root growth by precisely the same physiological mechanism. The idea that the anti-auxins stimulate root growth by antagonizing an endogenous auxin present in supra-optimal concentrations must, we think, be finally abandoned.

In a previous paper, where these views were first presented (Audus and Shipton, 1952), it was suggested that this proposed common physiological action of auxin and anti-auxin might be the antagonism of a natural endogenous inhibitor. This is of course not the only possibility. It is equally likely *a priori* that both types of compound may have a *direct* stimulatory action on the growth system of roots, in precisely the same sort of way that auxin is supposed to stimulate shoots and coleoptiles. The data which have been presented in this paper allow us to indicate which of these two theories is the more probable.

Much of the work of recent years on the relationships between growth response and external concentrations (c) of growth substances in both coleoptiles and roots (Kaindl, 1951; McRae and Bonner, 1953; Hellström, 1953; Bennet-Clark and Kefford, 1954) suggest that they can all be closely related by a formula of the following type:

$$\text{Response} = \frac{K.c}{A+B.c},$$

where K, A, and B are constants.

Now if $B = 1$, this takes the form of the equation for enzyme kinetics, which has been shown to fit with remarkable closeness the growth response of coleoptile sections to applied IAA (McRae and Bonner, 1953; Bennet-Clark and Kefford, 1954). If we write $A = 1$ and $K = B$, we have an expression directly derivable from the Freundlich adsorption isotherm, where the response would be directly proportional to the concentration of growth substance adsorbed at some cell interface. This equation, in the hands of

Hellström (1953), has been shown to fit, within limits of experimental error, the double response (i.e. stimulation followed in higher concentrations by inhibition) of roots to applied growth substances and also the antagonistic interactions of auxins and anti-auxins in root inhibitions. The assumption was made that growth substances affect growth as a result of their adsorption at some particular site in the cell (see also Kaindl, 1951). This adsorption centre may be an enzyme surface or it may not. There is no way of distinguishing between these two alternatives merely from the shapes of growth response curves.

The treatment of Hellström is well suited to the immediate problem since it is concerned with multiple adsorption patterns involving more than one adsorbate competing for the same adsorption centre. The only assumption which will be made, and this is a reasonable one, is that the effect of any compound, auxin, anti-auxin, or natural growth inhibitor, is some simple linear function of the amount of that substance adsorbed at the active centre. Let us now consider the interaction of applied growth substances expected on the two theories of growth stimulation suggested above.

Firstly, if stimulation is due to the antagonism of the action of a natural endogenous inhibitor, then there are three adsorbates in the system, the natural inhibitor, and the two interacting exogenous antagonists of this inhibitor, which we may call *A* and *B*. Since, in this theory, at the low concentrations considered, the two antagonists are not supposed to have any direct action on growth, then the growth *response* will be proportional to the quantity of inhibitor 'pushed off' the centres where it is adsorbed and exerting its inhibition. Let us assume that in the root section the inhibitor is present in such a concentration that a fraction a_1 of the adsorption 'mosaic' is occupied by it. Let us assume that when this 'mosaic' is saturated with the inhibitor, root section growth is reduced by a fraction H. Thus in the normal root section growing in 0·5 per cent. sucrose alone growth will be proportional to $(1-Ha_1)$. In the presence of the antagonists some of the inhibition sites will be occupied by these inactive substances and the fraction occupied by the inhibitor will be reduced to a_2. The growth will then be proportional to $(1-Ha_2)$ and the growth *response* brought about by the antagonism will be proportional to $H(a_1-a_2)$. Hellström gives a formula relating (a) to the concentrations of either one, two, or three adsorbates and to their 'constants of affinity' for the adsorption points. These expressions can be adopted for the present problem and rewritten in the forms:

$$a_1 = \frac{C_i/K_i}{1+C_i/K_i} \quad \text{and} \quad a_2 = \frac{C_i/K_i}{1+C_i/K_i+C_a/K_a+C_b/K_b},$$

where C_i, C_a, and C_b are the concentrations of inhibitor, growth substance A, and growth substance B respectively and K_i, K_a, and K_b their respective 'affinity constants'. An 'affinity constant' is equal to the concentration of the related substance which, when present alone, would cause half-saturation of the adsorption surface. Although we can have no idea of the values of H, C_i, and

K_i and can make only very approximate estimates of the probable values of constants K_a and K_b, yet we can easily predict the *form* of interaction this situation would give. For the sake of convenience it has been assumed that the sites are normally almost saturated with inhibitor, i.e. $C_i/K_i = 10$, but that at saturation, growth is reduced only by 25 per cent., i.e. $H = 0{\cdot}25$. Values of $K_a = 10^{-7}$ (estimated roughly from IAA response curves) and $K_b = 10^{-3}$ (estimated roughly from CNB response curve) were then used to construct the interaction surface shown in Fig. 9A, where the response is calculated as a fraction of normal growth. No account has naturally been taken of the onset of the inhibition phase at high concentrations (which Hellström has shown can also be fitted by similar formulae), but this does not affect the argument. It will be seen that the combination of two growth substances always results in an effect greater than that of either acting alone at the same concentration, but that there is an upper limit beyond which stimulation will not rise when all the inhibitor has been 'pushed off' its centre by either or both of the antagonists *A* or *B*. There is no mutual antagonism such as that observed in the experiments described above and this is a very strong objection to the natural endogenous inhibitor hypothesis. Further, such an hypothesis means that all active substances should give precisely the same degree of stimulation at optimum concentrations, which is certainly not so. (This applies equally well to the currently accepted theory of anti-auxin stimulation of root growth, where *auxin* is this natural endogenous inhibitor.) We cannot, however, apply this argument too rigidly since the maximum stimulation observed is probably a false maximum set by the rising stimulation curve and a falling inhibition curve (see Hellström, 1953). A low maximum stimulation is more likely to be

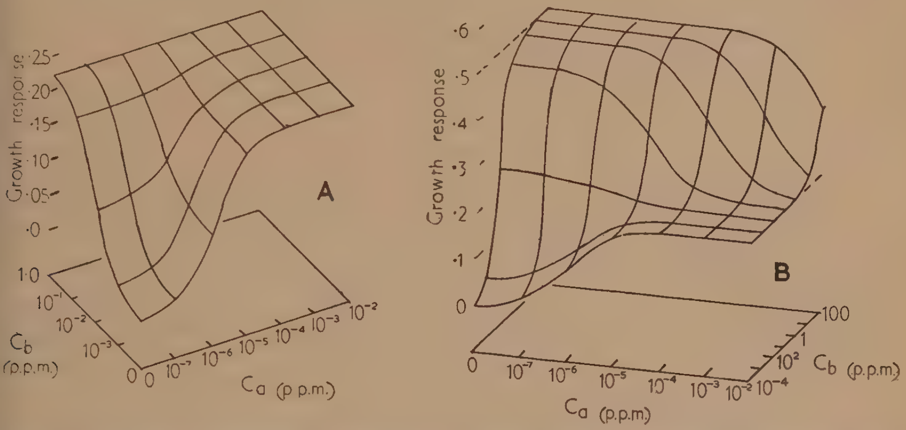

FIG. 9. Theoretical interaction curves for two growth substances active in the stimulation of extension growth of root sections, assuming growth control to be exerted by such substances when adsorbed at a specific growth centre.

A. When the stimulation is produced by the competitive antagonism of a natural endogenous growth inhibitor. B. When the stimulation is a direct action at the growth centre. (For further explanation see text.)

caused by an early onset of the inhibition at relatively low concentrations, the maximum possible stimulation never being reached.

The second possible mechanism of the growth stimulation is that there is no endogenous growth inhibitor and that the exogenous growth substances stimulate root growth by a direct action on the growth system. If again we assume that the stimulation is by molecules adsorbed at some protoplasmic surface, then we can again use the formula of Hellström. Here we are dealing with a system of two adsorbates, A and B, and, using the same symbols as before, the fraction of the area in the adsorption pattern occupied by A and B are respectively:

$$a_a = \frac{C_a/K_a}{1+C_a/K_a+C_b/K_b}$$

and

$$a_b = \frac{C_b/K_b}{1+C_a/K_a+C_b/K_b}.$$

Now Åberg (1952) has shown in his studies on the interactions of auxins in root growth that growth substance molecules have two distinct properties, an 'affinity' for the growth centre, which can be expressed by the above formulae, and an 'activity' at that centre, which can be regarded as the growth activity per adsorbed molecule. Using this concept he has shown that auxins with high 'affinity' and low 'activity' can behave as effective antagonists of more active auxins. On this basis we may give to our two growth substances activities A_a and A_b respectively, and the relevant growth response will then be proportional to the products $a_a A_a$ and $a_b A_b$. If therefore we make the assumption that the 'activities' of either A or B are not in any way modified by the presence of the other molecule, then we may write the total growth response due to the mixture as being proportional to $a_a A_a + a_b A_b$ or

$$\frac{C_a A_a/K_a+C_b A_b/K_b}{1+C_a/K_a+C_b/K_b}.$$

Again an interaction surface has been constructed from this formula making $K_a = 10^{-6}$ and $K_b = 10^{-2}$. The activities of A and B were taken arbitrarily at $A_a = 0{\cdot}25$ and $A_b = 0{\cdot}5$, i.e. B being twice as effective as a stimulant than A, per adsorbed molecule. The interaction surface calculated using these values is seen in Fig. 9B. Here it is clear that there is a definite antagonism of the action of the 'stronger' compound B by the 'weaker' one A. As the concentration of A is increased so the magnitude of the joint effect of $A+B$ is 'pulled down' towards the lower stimulation level corresponding to saturation of the growth centre by A. It will be seen that for any combination of concentrations the magnitude of the net effect on growth falls somewhere between the effects of the two compounds acting singly at those concentrations. It can never be smaller than that of the weakest growth substance acting alone.

On the whole this second picture fits the results obtained, particularly with those substances giving immediate growth responses, i.e. IAA × CNB and NMSA × CNB. In Fig. 7 it is clear that a combination of IAA at both stimulatory concentrations with optimum concentrations of CNB give effects that are of the same magnitude as the smaller effect given by IAA alone. Here IAA is the 'weaker' growth substance (corresponding with A) antagonizing the 'stronger' CNB (corresponding with B). In precisely the same way in Fig. 8 CNB, as the 'weaker' growth substance, is seen to antagonize the action of the 'stronger' NMSA.

It should be noted that if $A_a = A_b$, i.e. if the two growth substances have identical 'activities', then this second surface assumes virtually the same form as the first and the two theories are indistinguishable.

There is, however, one feature of the experimental interactions which is not explained by the theoretical treatment above. This is that combinations of low (suboptimal) concentrations of the strong growth substance with high (optimal) concentrations of the weak growth substance gives much lower growth responses than either acting alone, i.e. a marked *mutual* antagonism. This is clearly seen in combinations of 1 p.p.m. NMSA with 1, 10, and 100 p.p.m. CNB (Fig. 8). It is also seen, although not so clearly marked, in the IAA × CNB interaction with IAA at 10^{-5} and 10^{-4} p.p.m. and CNB at 0·1 and 1·0 p.p.m. (Fig. 7). Although many of the *individual* differences do not reach a satisfactory level of significance statistically, yet their consistent appearance in so many of the experimental interactions is very strong evidence that the effect is a real one.

Now it is possible to explain, purely in terms of previous concepts of growth action at an adsorption point, the mutual antagonism of two growth substances *present in optimal concentrations*. If we assume with Hellström that the decline of growth as the concentration is increased from this optimum is also due to adsorption, now at *another* site where inhibition *not* stimulation is effected, then it can be shown that a combination of two optimal concentrations will result in a net depression of growth below these maxima, i.e. an *apparent* mutual antagonism.

This is not so here, since the effects are seen in concentration combinations in which at least one component is very markedly suboptimal. At the moment it is difficult to see how best we may account for this mutual antagonism. Simple competition for a common centre of action will not explain it, as the above treatment shows. It is possible that the assumptions made in the theoretical analysis, that the 'activities' (i.e. A_a and A_b) of the competing substances are strict constants, is at fault. If the 'activity' of a molecule were in some way *reduced* by the presence of a competing molecule on a neighbouring adsorption site, then we could account for these excessively low responses to these mixtures.

CONCLUSIONS

There are two major conclusions that can be drawn from the foregoing experiments. Firstly, they give very strong support indeed to the earlier postulates that, as root growth stimulators, both auxins and their homologous anti-auxins are probably functioning *in identical ways* in the *same growth system*.

Secondly, they show that the idea of stimulation by the antagonism of endogenous growth inhibitors may have to be abandoned since the interaction patterns suggest a *direct* action of these compounds at the growth centres.

However, this cannot be the whole story of the action of these compounds in normal root growth. One major discrepancy which has to be explained is why PCIB should act as a very active growth stimulant in attached wheat roots (Burström, 1950) and yet be almost completely inactive in pea-root sections. It seems that the difference in response is due to specific plant reaction and that pea roots are insensitive to the stimulating action of PCIB, since observation on intact roots of pea seedlings have shown that, from the time of emergence of the radicle to the stage when laterals are beginning to form, PCIB exerts no stimulatory action on the elongation rate of the main root in any concentration. This adds yet another complication to the already confused picture and points to the necessity of extending the present root growth substance studies to a much wider range of plants.

Out of these results a purely practical point emerges. If these principles prove, on extension of studies to a wider range of compounds, to be of general application, it may well mean that we may have to modify radically the current ideas concerning the molecular structural requirements of growth substances active on roots. There is no doubt that we can no longer use root stimulation *alone* as an undeniable criterion of anti-auxin activity in a compound. This point of view is emphasized by the recent findings (Veldstra and Åberg, 1953) that some undoubted antagonists of the root growth-inhibiting action of auxins, i.e. some aryl- and aryloxy-acetic acids with bulky substituents on the α-carbon atom of the side chain, are completely without stimulatory action on root growth in low concentrations. PCIB falls into this category of root growth substance in the present experiments on pea roots.

The recent contributions of Åberg (1952) and Linser (1954) point the way to a more precise and intimate analysis of the structural requirements of active growth substances. As already foreshadowed by Veldstra (1947) almost a decade ago, the structural requirements for 'affinity' and for 'activity' must be very different. But so far we have tended to regard all types of growth in all kinds of organs as being ultimately controlled by the same growth system in which these compounds are acting directly.

It must now be recognized that the requirements for 'affinity' in one organ may be different from those in another, as would be expected if the adsorbing surfaces are proteins. It would indeed be surprising if the growth-controlling protein mosaic was precisely the same in the extending cells of both roots and shoots. Similarly we must be prepared to find that 'activity' requirements may also differ. In addition we have the further complication implicit in the analyses of Hellström that the same substance may have two different growth actions in the same organ, e.g. a stimulatory and an inhibitory, each involving a different 'affinity' and a different 'activity' at different adsorption sites. The structural requirements for these four aspects of the molecule, i.e. for maximum 'affinity' and 'activity' in both stimulation and inhibition respectively may well be different. The reasons put forward to explain the marked differences of behaviour of compounds in different growth tests have usually been sought in phenomena such as differential rates of adsorption, translocation, or inactivation, but we must not lose sight of the fact that the different growth centres may themselves have different structural requirements in the activating molecules.

ACKNOWLEDGEMENTS

The authors wish to acknowledge with appreciation and thanks the kind co-operation of Pal Chemicals Ltd., Professors Årne Fredga and R. L. Wain and Dr. Börge Åberg, but for whose generosity in donating the anti-auxins detailed on p. 331 this work would not have been possible.

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Some Aspects of Cobalt Metabolism by *Rhodymenia palmata* with Particular Reference to Vitamin B_{12} Content

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SUMMARY

The uptake of cobalt by *Rhodymenia palmata* from sea-water has been studied by the use of radio-cobalt 60. Periodic absorption and desorption associated with illumination have been found. A dependence on carbon dioxide concentration has also been established. No effect of the addition of antibiotics to the sea-water can be detected and no evidence of cobalt fixation as vitamin B_{12} has been obtained. Instead, another stable organic compound of cobalt has been discovered, differing from vitamin B_{12} in chromatographic and ionophoretic behaviour. The possible nature of the new compound is considered.

INTRODUCTION

SPECTROCHEMICAL analysis (Black and Mitchell, 1952) shows that, on a weight basis, marine algae contain cobalt in much greater concentration than their environment. This conclusion is confirmed by previous experiments in which the distribution of cobalt between algae and sea-water has been measured by radioactive tracer technique (Ericson, 1952). Since such a high concentration of cobalt is unlikely on a purely physical basis, some active concentration mechanism is probably involved. The synthesis of vitamin B_{12} by marine algae may provide a possible mechanism and the feasibility of this hypothesis is enhanced by the occurrence of vitamin B_{12} factors in seaweeds (Ericson and Lewis, 1953). In the present investigation an attempt has been made to confirm the concentration of cobalt as vitamin B_{12} by following the path of radioactive cobalt-60 from the sea-water to the algae and to its destination within the algae.

The investigation has been divided into two sections: (*a*) a study of the conditions affecting the uptake of cobalt and the preparation of the cobalt 60-labelled seaweed (at the Institute of Seaweed Research, Inveresk, Scotland, by R. S.), and (*b*) the extraction of the cobalt complex and proof of its identity or otherwise with vitamin B_{12} (at the Royal Institute of Technology, Stockholm, Sweden, by L.-E. E.).

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Experimental (*a*)

Culture conditions. All experiments were conducted with *Rhodymenia palmata* plants collected only a short time previously at North Berwick, south-east Scotland. These were immersed in 1 litre of filtered sea-water contained in 2-litre Pyrex aspirating bottles, which were maintained at 10° C. by externally circulating water cooled in a refrigerator. For 8 hours each day, unless otherwise stated, the plants were strongly illuminated by a bank of six 80-W. fluorescent tubes of the warm-white type, automatically controlled by a time switch. The tubes were housed in a common reflector suspended 2 ft. above the bottles and provided a light intensity of approximately 500 ft.-candles on their external surface. Aeration was accomplished by continuous suction from a water-pump.

Radioactivity determinations. These were made by type M. 6 (20th-Century Electronics) liquid counters with argon-alcohol filling. The count produced by these counters was not critical with respect to volume of solution being assayed once the central Geiger-Müller tube had been covered, but nevertheless accurate 10-ml. samples of sea-water were always removed from the aspirating bottles with a pipette, a syringe providing suction.

Associated electronic equipment consisted of a probe unit type 1014A, scaling unit type 1009A, and potentiometer and power supply unit type 1007.

Absorption experiments. For the experiments whose results are shown in Figs. 1 (*a*) and (*b*) two *Rhodymenia palmata* plants were immersed in sea-water in aspirating bottles as described above. To each bottle was added 0·01 ml. of a solution of cobaltous chloride, containing 7·7 μg. total cobalt and 17·5 μC. total activity. A further addition of 10 μg. of streptomycin and 10 μg. of chloromycetin per ml. of sea-water was made in the experiment of Fig. 1 (*b*) both at the start and after 8 days. As an additional precaution, the sea-water for this experiment was boiled prior to use. Previous work by Ericson and Lewis had already shown the value of antibiotics in killing most bacteria living on seaweed and in sea-water.

At various time intervals counts were taken on 10-ml. samples of the sea-water in each aspirator to which the samples were again returned after use.

An initial plotting of results revealed a large fluctuation which could not be accounted for by experimental error. Counts taken on the sea-water later in the day were found to be consistently lower, corresponding to a higher cobalt uptake, than those in the morning. Light intensity was the only factor fluctuating in such a periodic manner and from Fig. 1 (*a*) and (*b*) the consistent agreement between light periods and cobalt movement can be readily observed. A similar observation on the movement of caesium has already been found (Scott). In confirmation of the accumulation of cobalt during the light periods, the plants of experiments 1 (*a*) and (*b*) were deprived of light by wrapping the experimental bottles in black cloth. The periodicity in the results was at once destroyed and appreciable desorption of cobalt occurred (after 211 hours, Fig. 1).

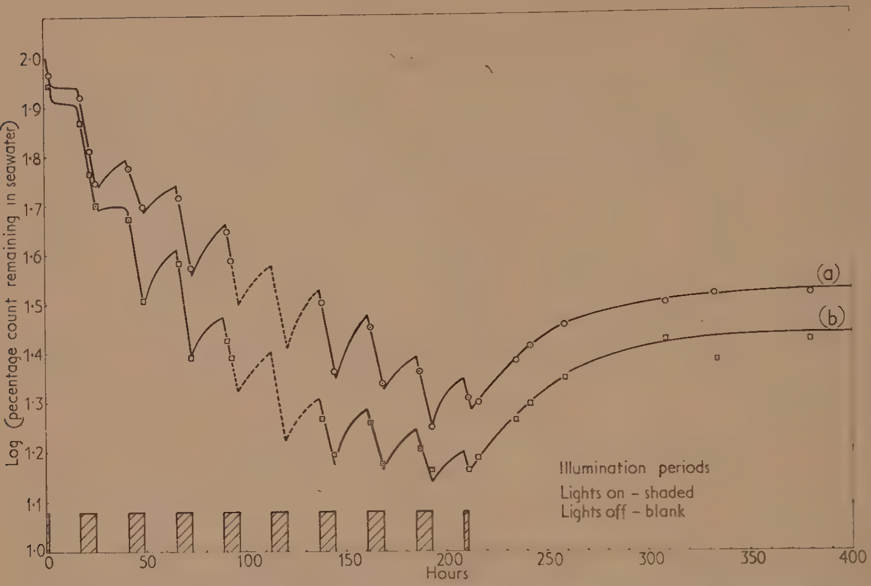

FIG. 1 (*a*) and (*b*). Uptake of cobalt 60 from sea-water by *Rhodymenia palmata*. Plant wet weights were 9·29 g. and 10·32 g. in (*a*) and (*b*) respectively. In (*b*) a mixture of 10 μg. streptomycin and 10 μg. chloromycetin per ml. of sea-water was added on the first and eighth days.

Although the above experiments were conducted at a cobalt level appreciably higher than that found in sea-water under natural conditions, it was tacitly assumed that results would be substantially the same irrespective of added cobalt content. However, an attempted proof of this assumption produced the results shown in Fig. 2. During this experiment as small a cobalt concentration was employed (approximately 1 μg./l.) as the specific activity of the tracer would permit. The detection of the periodic lighting effects was then only just realizable, the curve assuming the form of an absorption of cobalt or of an exchange of radioactive and stable cobalt of similar type to that found by Kelly and Baily (1951) for iodine exchange in *Ascophyllum nodosum*.

It was thought that, if cobalt absorption had occurred, the cobalt content remaining after some days in the sea-water would be extremely small and insufficient to satisfy the cobalt requirements of the plant. In these circumstances, cobalt might not desorb during darkness because its maximum possible level had not been reached during illumination. Thus, periodic fluctuations in cobalt level might be obscured.

An alternative explanation could be advanced. The curve might simply be an exchange of stable and radioactive cobalt, the final radioactive cobalt level in the sea-water being so small (actually of the order of a few counts per minute per 10 ml. sample of sea-water) that the periodic effects would be difficult to detect. Such an exchange is readily conceivable. Indeed, during the periodic absorptions and desorptions of cobalt already observed, radioactive and stable cobalt must inevitably become thoroughly mixed so that ultimately the ratio of stable to radioactive cobalt in the plant must equal the same ratio in the sea-water. Then only can the variation in radioactive cobalt level in the sea-water accurately reflect cobalt movements.

In proof of the exchangeability of the cobalt, excess of stable cobalt (2 mg./l.) was added in the form of chloride to the above experiment (Fig. 2) at $334\frac{1}{2}$ hours. Radio-cobalt already absorbed by the plant was rapidly desorbed to some extent, owing to its substitution by predominantly stable cobalt. The existence of the exchange phenomenon was, therefore, confirmed. On this basis, the flat initial portions of the uptake curves of Fig. 1 (*a*) and (*b*) can be attributed to the compensation of removal of radioactivity from the sea-water, owing to ionic exchange, by a gain in actual cobalt content in the sea-water (partly radioactive), owing to desorption during darkness.

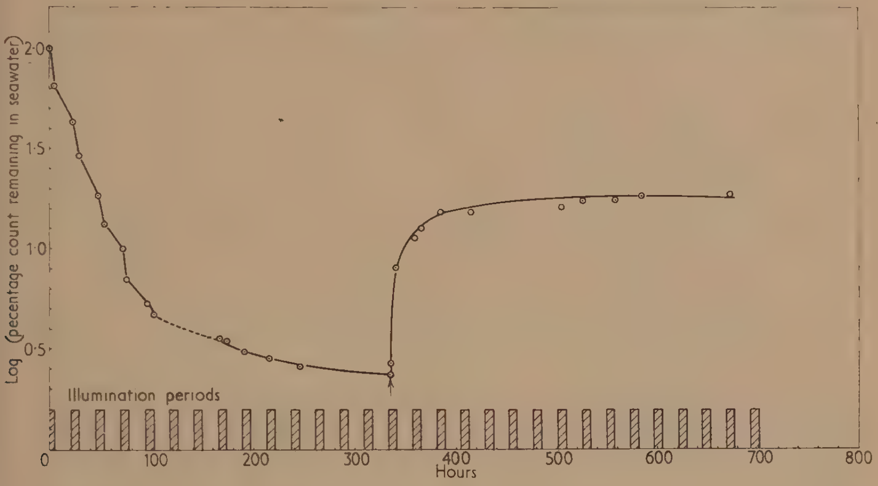

FIG. 2. Uptake of cobalt 60 from sea-water by *Rhodymenia palmata*. Plant wet weight was 7·94 g. and added cobalt content associated with tracer was approximately 1 μg. per litre. At the arrow a further addition of 2 mg. cobalt (as cobaltous ion) was made.

It has been deduced that periodic illumination will facilitate cobalt exchange by inward and outward movement of cobalt. Further, the actual cobalt content of a plant under such conditions will be higher than a corresponding plant kept entirely in the dark, as shown by Fig. 1 (*a*) and (*b*). Both these facts will contribute to a much greater observed uptake rate of radioactive cobalt in the light, as measured by the methods previously described. This was indeed proved to be the case, as shown in Fig. 3.

An elegant method of illustrating the more rapid exchange of cobalt and higher cobalt level during illumination with *Rhodymenia palmata* is shown by

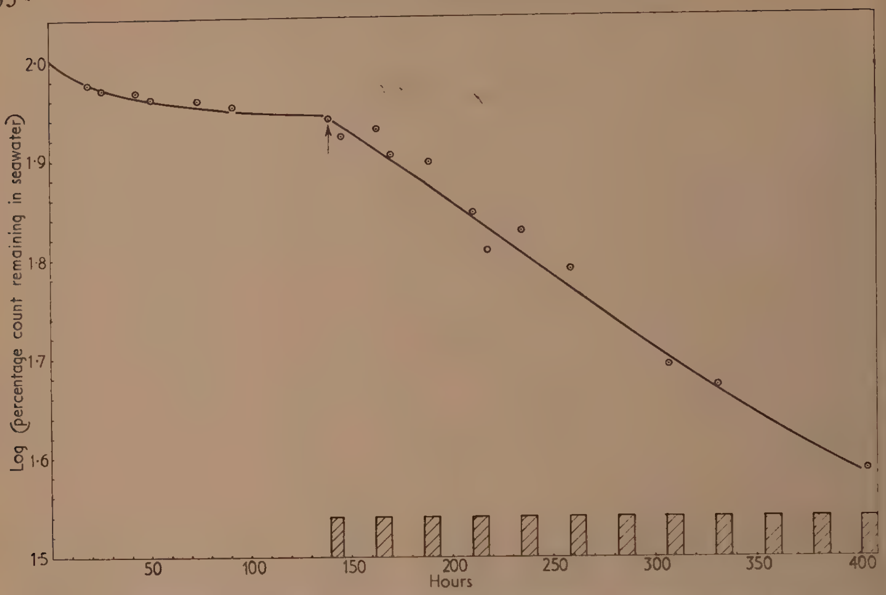

FIG. 3. Comparison of uptake of cobalt 60 by *Rhodymenia palmata* during darkness and periodic illumination. Plant wet weight was 4·70 g. Periodic lighting effects have been ignored in the drawing of the latter portion of the graph.

the autoradiograph (Fig. 4) of a plant kept in a fixed position in sea-water containing cobalt 60 and illuminated in one specific circular area, the remainder being blacked out. Similar results with other isotopes have already been achieved (Scott). An activity of approximately 20 μC. of cobalt per litre was employed. After 5 days the plant was removed, placed on a glass sheet, and covered with sheets of filter paper on which pressure was applied by lead blocks. The autoradiograph was subsequently obtained by leaving the plant in direct contact with an Ilfex double-coated no-screen X-ray film for 18 hours. Fig. 4 is a contact print from the autoradiograph. The totally white area is that originally illuminated.

Examination of the painted plate used to mask the greater portion of the plant revealed that some of the spurious white spots were derived from light leakages. Cobalt absorption by colonies of bacteria or epiphytic growth might be responsible for the remainder, but protuberances from the surface of the plant could also give rise, in these instances, to a misleading distribution of activity.

Desorption experiments.[1] For these experiments two plants of wet weights (*a*) 8·29 g. and (*b*) 8·55 g. respectively, which had absorbed radioactive cobalt in periodic light for 14 days while maintained in the same sea-water, were each washed twice with natural sea-water to remove surface activity and transferred to separate aspirating bottles containing 1 litre of sea-water. One bottle was subjected to periodic illumination (8 hours per 24 as previously specified),

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[1] The term 'desorption', as used here, denotes release of radioactivity. It is not used as the antonym of adsorption.

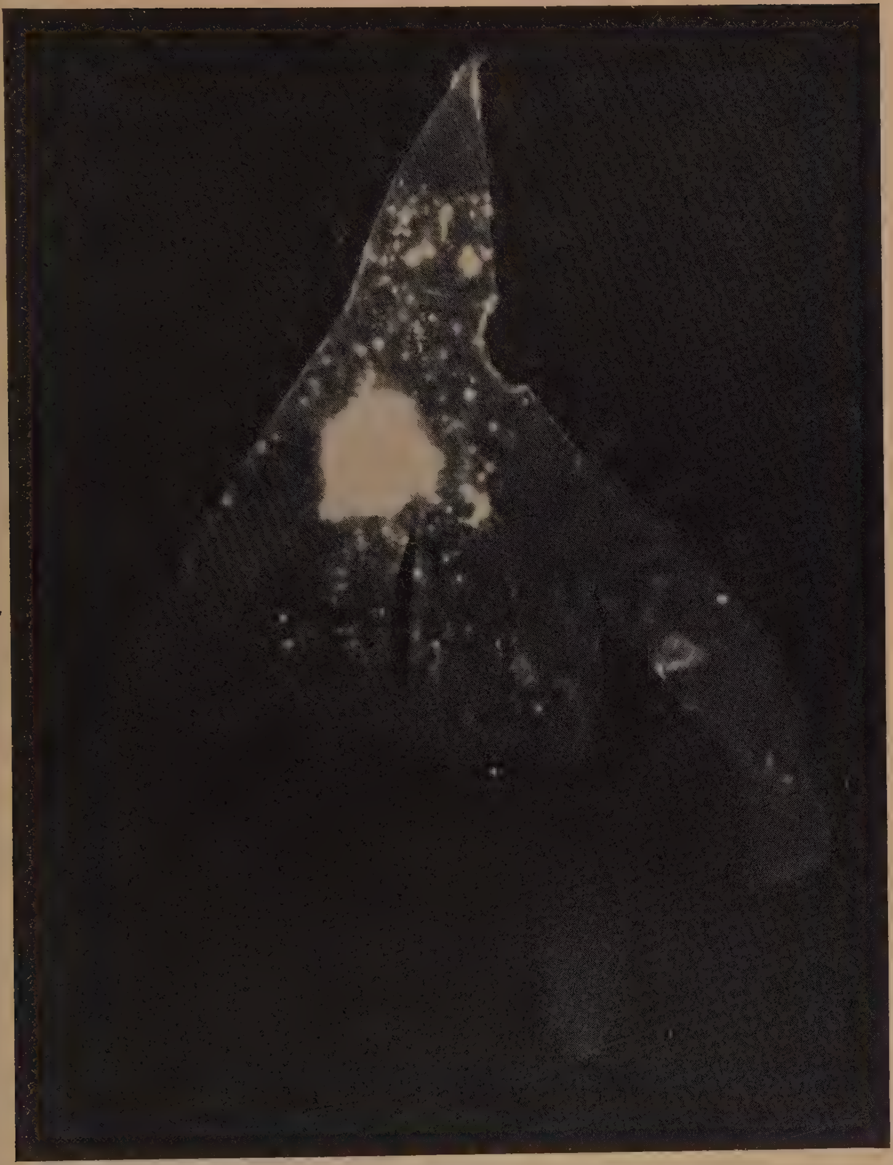

FIG. 4.

while the other was kept in darkness. Each was aerated as before. The results of these experiments are shown in Fig. 5 (*a*) and (*b*).

In general, desorption of activity can be attributed to an exchange of radioactive cobalt in the plants with stable cobalt in the sea-water, and to any readjustment of the cobalt distribution. Although the rate of cobalt exchange ought to be faster in periodic light than in darkness, final values of radioactivity in the sea-water should be closely similar if no other factors operate. In fact, comparison of Fig. 5 (*a*) and (*b*) reveals a significantly greater desorption of cobalt in the dark. Previous experiments point to the readjustment of the total cobalt level in the dark, by comparison with the light, as the discriminating factor.

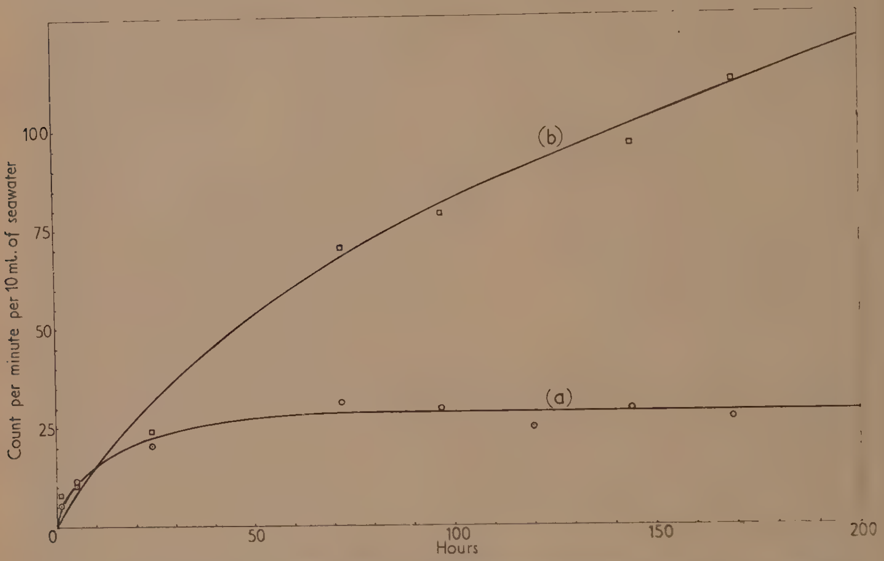

FIG. 5. Desorption of cobalt 60 from two *Rhodymenia palmata* plants. (*a*) in periodic light (8 hours per day) and (*b*) in darkness. The plants had been previously maintained in the same sea-water with 8 hour per day illumination.

Effect of carbon dioxide concentration. Owing to the relationship of cobalt absorption to illumination, it might also be thought to be dependent on photosynthesis. Removal of carbon dioxide from the aerating air, by arresting photosynthesis, ought also to depress cobalt absorption. For the experiment of Fig. 6, carbon dioxide was removed by means of a tower containing 'Sofnolite' (self-indicating soda-lime). Contrary to expectation, under these conditions, radioactive cobalt was more rapidly absorbed and the resulting cobalt concentration greater than under normal conditions, apparently by inhibition of the dark release process. On readmitting air containing its normal percentage of carbon dioxide, a readily detectable cobalt desorption occurred. It should be mentioned that periodic effects were obviated in the latter portion of the experimental curve, by making radioactivity assays as near as possible to a fixed time each day.

As a further indication of the effect of carbon dioxide on cobalt level, an experiment was conducted initially in duplication of that of Fig. 1 (*a*). At $239\frac{1}{2}$ hours, however, a mixture of 5 per cent. carbon dioxide and 95 per cent. air from a cylinder replaced the incoming ordinary air to the aspirating bottle. Immediately, cobalt desorption started to occur, despite the simultaneous pposing tendency of absorption owing to illumination. At a later stage 407½ hours) ordinary air was readmitted, whereupon a readjustment of cobalt evel took place. The plant showed no visible signs of damage attributable to he high carbon dioxide level although on another occasion, when pure oxygen vas employed, there was a rapid change in colour from red to brown and no ositive results could be achieved.

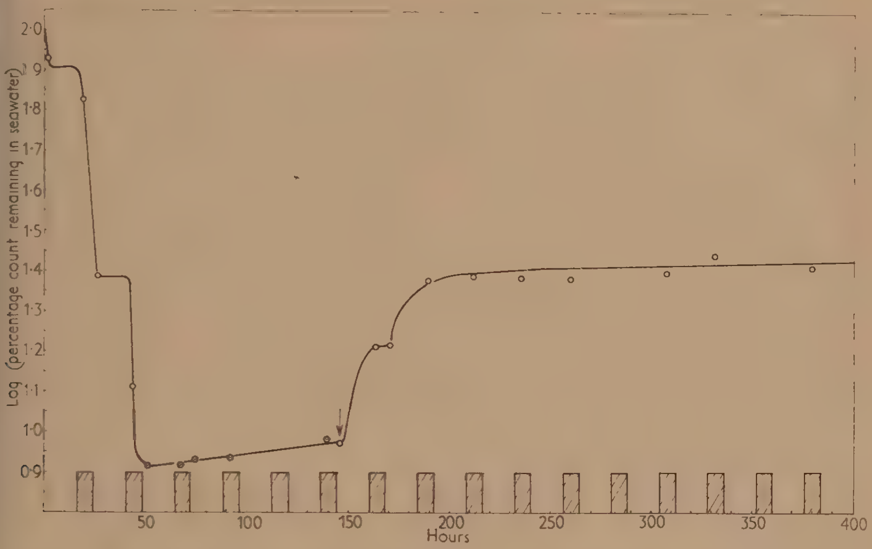

FIG. 6. The effect of carbon dioxide deficiency on the uptake of cobalt 60 by *Rhodymenia palmata*. Plant wet weight was 8·29 g. At 145½ hours (arrow) air with its normal carbon dioxide content was readmitted.

Experimental (*b*)

Preparation of samples. Rhodymenia palmata plants of wet weights 24·91 and 24·42 g. respectively were each added to 1 litre of sea-water containing 36·6 μg. radioactive cobalt and approximately 87·5 μC. activity. To the second experiment 10 mg. each of streptomycin and chloromycetin were added both on the first and on the eighth days. As an additional precaution, for the experiment whose results are shown in Fig. 1 (*b*) the sea-water was boiled, cooled, and re-aerated. After 17 days both plants were removed, dried for 16 hours below 50° C., and dispatched to Stockholm. In each case, at the end of the experiment only about 12 per cent. of the original activity remained in the sea-water.

Seaweed samples from each of these experiments and a natural sample not cultivated at all in the laboratory were dried and ground. Each was individually boiled twice in a mixture of phosphate buffer and acetone containing cyanide, this procedure having been found by Ericson and Lewis (1953) to be most efficient for the release of B_{12} factors from seaweed. The algal material was shaken with a few millilitres of water and the combined extracts, after evaporation of the acetone, were analysed for vitamin B_{12} by the use of *Escherichia coli* 113–3 (Davis and Mingioli, 1950) in the agar cup plate method described by Diding (1951) with the following results:

Rhodymenia palmata under the following conditions	Vitamin B_{12} content (μg. per g. of dry weed)
1. 17 days in antibiotic sea-water . . .	0·23
2. 17 days in natural sea-water. . . .	0·22
3. Natural sample	0·24

Bioautography, again using *Escherichia coli*, revealed that the biological activity was due to vitamin B_{12} (cyanocobalamin). No other B_{12} factors could be detected. The table shows that weed which had accumulated radioactive cobalt possessed no higher vitamin B_{12} content than natural weed harvested at the same place and time. Values for those samples harvested in mid-April 1953 were higher than those previously reported for the same species harvested in July 1952, implying that the vitamin B_{12} content of algae varies with the season (Ericson and Lewis, 1953). In the sample from July 1952 small amounts of other vitamin B_{12} factors were observed.

Paper chromatography and ionophoresis were employed to investigate the fate of the cobalt absorbed by the seaweed. For the chromatographic separation of a mixture of *sec*-butanol, saturated with water and containing 3 per cent. acetic acid and 2·5 mg. sodium cyanide per 100 ml., was used. Other solvent systems which were tried failed to produce a better separation. Ionophoresis was carried out in a phosphate buffer, having a pH of 4·6, on free hanging paper with a potential difference of 700 V.

On the chromatograms the sites of radioactivity and vitamin B_{12} activity were determined. These did not coincide. The radioactivity was not associated with simple cobalt ion since this substance, when placed on the same initial spots on chromatograms as extracts of *Rhodymenia palmata* derived from plants maintained in natural and antibiotic sea-water, separated from the radioactive components of the extracts (and also from vitamin B_{12}) as shown in Fig. 7. In confirmation of these results, when examined by ionophoresis, radioactive cobalt ion migrated towards the cathode, while the radioactivity from both extracts moved towards the anode (Fig. 8).

As a further proof that *Rhodymenia palmata* had not utilized cobalt during the culture period for the synthesis of vitamin B_{12}, 4·7 mg. of 'carrier' crystalline vitamin B_{12} was added to an extract of the plant which had been maintained in 'antibiotic' sea-water and had a total vitamin B_{12} content of 230 μg. Vitamin B_{12} was isolated from the mixture by treatment with ion-exchange resin IRC–50, chromatography on alumina and, subsequently, by distribution between *n*-butanol and water and between phenol and water. The vitamin, which was finally precipitated by adding acetone, was slightly radioactive. On ionophoresis, the radioactive material, moving towards the anode, separated from the red spot due to vitamin B_{12}, which moved towards the cathode. This red spot had no detectable radioactivity. Owing to the small amount of

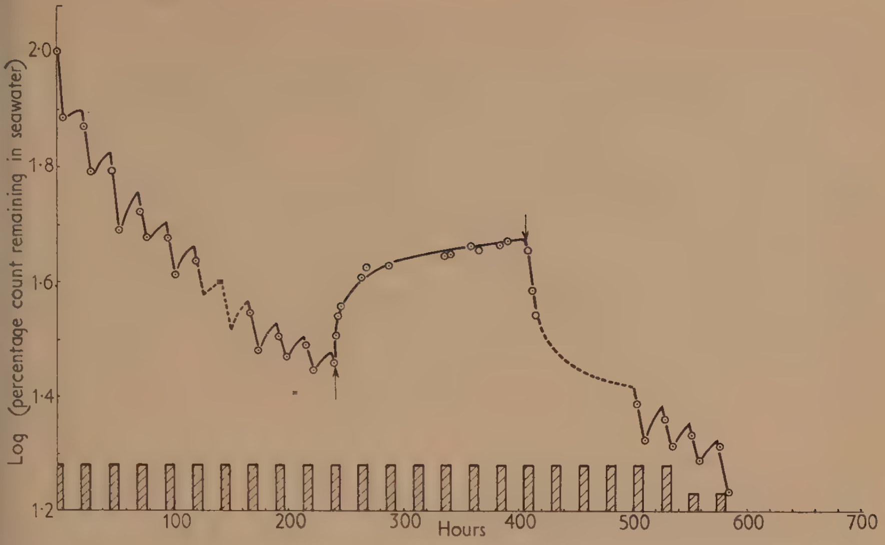

FIG. 7. The effect of carbon dioxide on the uptake of cobalt 60 by *Rhodymenia palmata*. Plant wet weight was 9·07 g. At 239½ hours (first arrow) a mixture of 5 per cent. carbon dioxide and 95 per cent. air was used for aeration. From 406 hours (second arrow) air with its normal carbon dioxide content was employed.

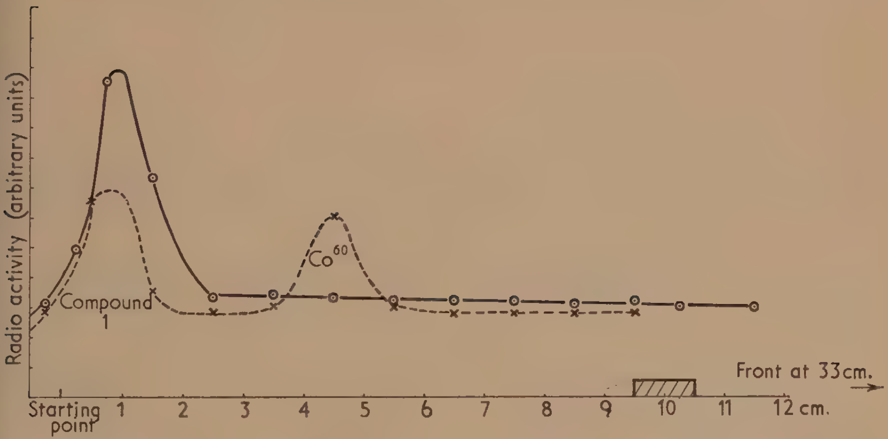

FIG. 8. Distribution of radioactivity on a paper chromatogram of an extract of *Rhodymenia palmata* maintained in sea-water containing cobalt 60 for 17 days (full line) and of the same sample with cobalt 60 directly added before extraction (dotted line). The shaded area is the site of vitamin B_{12} activity.

vitamin B_{12} present, recrystallization, as a means of freeing the vitamin from contaminating radioactivity, was impracticable.

To ensure that the radioactive cobalt complex was not an artefact produced during extraction, radioactive cobalt ion was added to the solution used for the extraction of the algae and the whole extraction procedure carried out as previously described. When the extract was examined the cobalt originally added was still present as the free ion.

All the evidence outlined above confirmed that the cobalt absorbed by the seaweeds was no longer present in its simple ionic form but as part of a complex, readily distinguishable from vitamin B_{12}. Ionophoretic evidence suggested that this complex contained more than one cobalt compound, and for convenience the main fraction is referred to below as fraction I.

Fraction I proved to be very stable. On hydrolysis in N. HCl or N. NaOH at 120° C. for 2·5 hours, very little destruction took place. Only traces of free radioactive cobalt could be detected in the solutions thus treated. On hydrolysis in 6 N. HCl at 100° C. for 24 hours, however, it was completely broken down and all the radioactivity released as free cobalt ion.

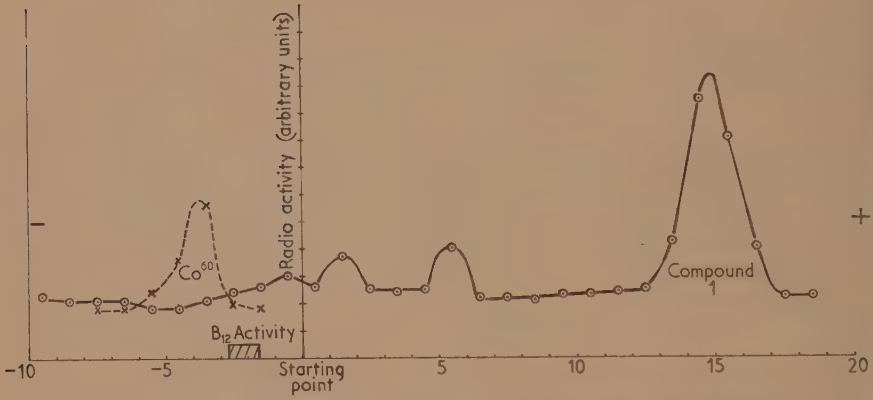

FIG. 9. Ionophoretic separation of compound 1 from an extract of *Rhodymenia palmata* plants maintained in sea-water containing cobalt 60 for 17 days (full line) showing the pronounced displacement of added cobalt radioactivity (dotted line) and vitamin B_{12} biological activity (shaded).

Ascorbic acid was also efficient in releasing cobalt from fraction I. Even a 0·03 M. solution of ascorbic acid was sufficient to liberate radioactive cobalt quantitatively on autoclaving at 120° C. for 1 hour. This is of interest since Frost *et al.* (1952) have shown a similar cobalt release from vitamin B_{12b} and B_{12} by ascorbic acid. Oxalic acid under identical conditions failed to release cobalt from fraction I. The effect of ascorbic acid can hardly be attributed to its reducing properties since catalytic reduction of extracts containing fraction I over a platinum catalyst also failed to liberate any ionic cobalt.

Enzymatic conversion of this fraction was also attempted. Pepsin at pH 2·5, trypsin at pH 8·0, and the enzyme mixture of a chicken pancreas homogenate all produced no modification of the ionophoretic properties. On the other hand, bacteria isolated from sea-water (bacterium No. 3, Ericson and Lewis, 1953) when cultivated in the presence of fraction I, proved to be capable of converting this substance into some other cobalt-containing compound. Little or no radioactive cobalt ions could be detected in the fermentation broth.

DISCUSSION

Consideration of the experimental results makes it necessary to conclude that *Rhodymenia palmata* does not accumulate cobalt for the specific synthesis of vitamin B_{12} and that bacteria are not principally concerned with cobalt accumulation.

Quantitative considerations all point to some independent method of cobalt fixation. For instance, from the experiment shown in Fig. 1 (*a*) a concentration ratio of 1,854·5 can be calculated at 193 hours on the basis of the dry weight of the plant (2·56 g.). Assuming for the moment that the natural cobalt content of sea-water is 0·3 μg./l. or $0{\cdot}3 \times 10^{-9}$ g./g. of sea-water approximately, the cobalt content of the marine alga must have been $0{\cdot}556 \times 10^{-6}$ g./g. of dry seaweed. It should be pointed out that, in deriving this cobalt content of the alga, two figures of doubtful validity have been employed. The figure of 0·3 μg. l. for the cobalt content of sea-water is almost certainly high,[1] but the corresponding concentration factor is undoubtedly low. Factors of the order of 25,000 have been achieved with minute radioactive cobalt additions and in all probability such factors more closely represent natural conditions. Nevertheless, the resulting figure for the cobalt content of the alga is corroborated by the work of Black and Mitchell (1952) who arrived at the same order of result for other species. Now, since only about 4·5 per cent. of vitamin B_{12} consists of cobalt, according to Brink *et al.* (1949), the expected B_{12} content of the alga will be

$$\frac{100}{4{\cdot}5} \times 0{\cdot}556 \times 10^{-6} \text{ g./g. dry material}$$

or approximately 12·5 parts per million. The measured B_{12} content is only about 0·25 parts per million, however, showing the necessity for some other mode of cobalt fixation. On the evidence presented here, cobalt appears to be fixed principally in fraction I and not as vitamin B_{12}.

Relatively few compounds of cobalt, apart from vitamin B_{12} and related factors, have been found in nature. Cobalt complexes formed *in vitro* with amino-acids have been described by Burk, Hearon, Caroline, and Schade (1946) and peptides by Gilbert, Otey, and Price (1951). Cobalto- and cobalti-porphyrins have also been obtained (see Lemberg and Legge, 1949).

From the evidence of known cobalt complexes, two possible methods of co-ordinate linkage of the cobalt in fraction I can be envisaged. If the cobalt were co-ordinated with the $—NH_2$ and/or —SH groups of one or several amino acids or peptides (Orten and Bucciero, 1948; Gilbert, Otey, and Price, 1951), leaving only the carboxy groups free to dissociate, then the strongly anionic character of fraction I could be explained. On the other hand, the similar effect of ascorbic acid both on fraction I and on vitamin B_{12}, for which a porphyrin-like structure has been proposed by Beaven, Holiday, Johnson, Ellis, and Petrow (1950), suggests a comparable structure for the complex.

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[1] Black and Mitchell give the figure $< 0{\cdot}3$ μg. per litre. In a private communication Dr. Mitchell states that this is a lower limit of detection.

The inability of enzymes to cause any significant changes in fraction I is i agreement with both these hypothetical formulations, although the secon appears to have the more interesting implications in view of the importance o similar structures in essential plant substances such as the chlorophylls an cytochromes. Fraction I is not, however, associated with chlorophyll in th alga since it is unaffected by alcholic extraction. Recent work (by R. Scott reveals that in sufficiently acid solutions the compound acquires a positiv charge. Such an amphoteric behaviour indicates an amino-acid, peptide, o protein complex in accordance with the first structure. An attempt to extrac the complex by butanol after hydrolysis of the weed residue by bariu hydroxide met with little success.

The question of the dominant effect arising from the lack of carbon dioxid requires further study. pH measurements on sea-water under conditions o carbon dioxide deficiency exactly comparable with those of the present experiments both in the presence and absence of algae showed a pH shift from 7·9–8·0 to 9·0–9·1. In the presence of algae the change occurred in two days but required about four days without algae. Presumably, the algae accelerated the transition by utilization of dissolved carbon dioxide. During aeration by 5 per cent. carbon dioxide the pH dropped from a starting value of 7·9 to 6·4 after six days. These pH changes in the surrounding sea-water, however, may not reflect the changes occurring within the algae where further changes must also occur owing to the normal processes of respiration and photosynthesis. The question remains whether the effect of carbon dioxide deficiency and excess is directly or indirectly linked with cobalt uptake or influences cobalt accumulation by altering the pH of some enzyme systems or the dissociation of some reactant.

Disregarding these latter possibilities and assuming that carbon dioxide has a direct effect, one can account for the existing facts by asserting that, as a direct or indirect result of photosynthesis, substances are produced which react with cobalt ion with the formation of the stable complexes. The complexes are probably present in very small concentration and in a state of continuous turnover.

The inverse relationship between cobalt accumulation and carbon dioxide supply implies that the latter reacts with the complexes with liberation of free cobalt ion. In light, carbon dioxide is removed by photosynthesis, thus allowing principally compound I to be produced with consequent cobalt accumulation. In darkness, carbon dioxide is available owing to respiration and acts on compound I, releasing cobalt as observed.

By removing carbon dioxide both by photosynthetic and chemical means the plant is deprived of its ability to regenerate cobalt as free ion. Its interna cobalt content, therefore, increases. In the dark, during chemical removal o carbon dioxide, the small quantity produced by respiration is lost so rapidl that regeneration of cobalt as free ion is thereby prevented. Excess carbo dioxide, as anticipated on the above hypothesis, facilitates cobalt releas and inhibits net cobalt uptake. The comparable cobalt percentages release both by excess carbon dioxide and by darkness also seem to support these views.

The small naturally occurring vitamin B_{12} content of the alga relative to its large cobalt accumulation and the absence of radioactive vitamin B_{12} production in the experiments reported seem to infer that, normally, vitamin B_{12} is synthesized by *Rhodymenia palmata* by a slow alternative route, perhaps by bacteria living in the surrounding sea-water or on the surface of the plant. Ericson and Lewis (1953) favour these views which are supported by evidence obtained by Ericson (1952) with cobalt 60—labelled vitamin B_{12}. These authors suggest that vitamin B_{12}, originating by bacterial synthesis, is subsequently accumulated by marine algae.

ACKNOWLEDGEMENTS

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The Deamidase of Groundnut Plants (*Arachis hypogaea*)

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SUMMARY

The deamidase enzyme system present in extracts of groundnut plant tissues has been studied. The distribution of the enzyme within the different organs of the plant were determined, and some of the properties of the enzyme present in leaf extracts are recorded. From its substrate specificity the enzyme is probably best regarded as a γ-methyleneglutaminase, although it catalyses the hydrolysis of glutamine at about one-fifth of the rate of γ-methyleneglutamine. The enzyme may play an important rôle in the over-all nitrogen metabolism of the plant, as well as controlling the relative concentrations of γ-methyleneglutamine and γ-methyleneglutamic acid in the different organs. The properties of the enzyme have been compared with those recorded in the literature of glutaminases and asparaginases, and certain common features are apparent. The new deamidase was, however, more stable to certain denaturing treatments than were the other types of deamidase.

INTRODUCTION

Two related unsaturated amino-acids were isolated from groundnut plants by Done and Fowden (1952), and were provisionally identified as γ-methyleneglutamic acid (γ-MGA) and γ-methyleneglutamine (γ-MG). These structures have recently been confirmed by comparison of the natural products with γ-MG and γ-MGA prepared by rational chemical synthesis (Wailes, Whiting, and Fowden, 1954). These substances are far from being universally distributed in plants, but have been identified in tulips (Zacharius, Pollard, and Steward, 1954) and hops (Tatchell and Harris, 1953), where, as in groundnuts, they occur in considerable quantities.

The distributions of γ-MG and γ-MGA within the different organs of groundnut plants indicate that these compounds may play an important role during the processes of cotyledon proteolysis, nitrogen translocation, and meristematic protein synthesis which accompany the normal growth of young seedlings (Fowden, 1954*a*). Several enzyme systems utilizing either γ-MGA or γ-MG are known. Two of these, a decarboxylase and a transaminase, have been the subjects of previous publications. γ-MGA is decarboxylated by extracts of a number of plant species, but always at a rate slower than that determined under similar conditions for glutamic acid (Fowden, 1954*b*). This decarboxylase activity, probably to be identified with glutamic decarboxylase is low in groundnut plants, and may therefore account for the very smal concentrations of α-methylene-γ-amino-butyric acid (the decarboxylation

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product of γ-MGA) present in the plants (Fowden and Done, 1953*a*). Transaminase systems, present in various tissues of groundnut plants, rapidly utilize γ-MGA (Fowden and Done, 1953*b*), and could be responsible for the final stage in the synthesis of this amino acid from nitrogen-free precursors during the early growth of seedlings.

Brief mention has previously been made (Fowden, 1954*a*) of the presence of a third enzyme, responsible for the deamidation of γ-MG to γ-MGA, which appeared to be very active in extracts of groundnut leaves, but to be absent from the roots. Since this enzyme will be one of the factors determining the γ-MG/γ-MGA concentration ratios existing in different organs of the plant, a more detailed study of its distribution and properties has been made. The results obtained form the experimental portion of the present paper, in which suggestions are also made regarding the manner in which the deamidase may be integrated into the general system responsible for the nitrogen metabolism of the plant.

GENERAL EXPERIMENTAL PROCEDURE

Preparation of enzyme extracts. The groundnut plants used were grown under glass at about 30° from a single batch of seed. Tissue extracts were prepared by grinding the fresh plant material with half its fresh weight of 0·05 M. potassium phosphate buffer of appropriate pH and a little fine quartz sand until a smooth paste was obtained. This was strained through muslin, and the filtrate centrifuged for 5 minutes at 1,000 g. The supernatant liquid was then enclosed in a collodion bag and dialysed with slow continuous shaking against two lots of 250 ml. of 0·05 M. phosphate buffer for 4 hours at 0°. At the end of this period chromatographic examination of the dialysate showed it to be free of amino acids. The extracts so prepared were used directly as sources of deamidase activity in many of the experiments, whilst in others the dialysate was subjected to further treatments (freeze-drying, heat denaturation, &c.), which will be described in detail later when particular experiments are being considered.

Conditions of enzymic amide hydrolysis. All reaction mixtures contained the amino-acid amide substrates at an initial concentration of 0·015 M. In cases where the amide was a synthetic *dl*-racemate, the initial concentrations were doubled, in order to maintain the *l*-form at 0·015 M. The volumes of the dialysed extracts used as sources of deamidase activity were adjusted such that they formed half the volumes of the final mixtures. The pH's at which the reactions were performed were determined using a micro glass electrode, and could be varied by using buffers of different pH during the extraction procedure. Deamidation was carried out in a thermostatic bath controlled at 25°, and the reactions were stopped after the required times by the addition of 3 volumes of ethanol.

Measurements of reaction rates. Sensitive methods are available for measuring both the products of amide hydrolysis. Ammonia may be measured colorimetrically after Nesslerization, whilst the acidic amino-acid produced can be determined chromatographically. Ammonia, however, tends to be lost from alkaline solutions, and, since a number of the experiments were performed at pH values above 7, errors in determining the percentage of reaction based on measurements of ammonia formed may have been appreciable, especially when slower reactions at high pH's were being studied. The rates of deamidation have therefore been obtained by determining the amounts of dicarboxylic amino-acid produced. The acidic amino-acids present in 100 μl. of reaction mixture (in 400 μl. after the reaction had been stopped by the addition of ethanol) were first separated from residual amide on Whatman No. 3MM filter-paper chromatograms developed for 18 hours in the *n*-butanol-acetic acid mixture of Partridge (1948). They were then quantitatively estimated by the method of Fowden (1954*a*), or, in cases where less than 5 per cent. amide hydrolysis had occurred, semi-quantitatively by comparing the intensities of the ninhydrin-developed spots with those of a series of spots produced from graded standard amounts of the appropriate amino-acid.

The total nitrogen contents of the enzyme extracts were determined by the micro-Kjeldahl method, and the rates of reaction quoted later are expressed on a common basis as μg. amide-nitrogen hydrolysed per hour per mg. total nitrogen (excluding the added amide-nitrogen) present in the reaction mixtures.

RESULTS

The normal source of the deamidase used in the experiments designed to study the properties of the enzyme was leaves, since they yielded the most active extracts. Such extracts hydrolysed γ-MG rapidly and effected complete conversion of the natural amide to γ-MGA in about 3 hours. Synthetic γ-MG was never hydrolysed beyond 50 per cent. completion, indicating that the *d*-form was not attacked by the enzyme. The possibility of complete hydrolysis of the natural amide was taken as evidence that it was an *l*-form. Fig. 1 shows the results of a typical experiment relating the percentages of the γ-MG hydrolysed with time. Since, under the conditions employed, the rate appears

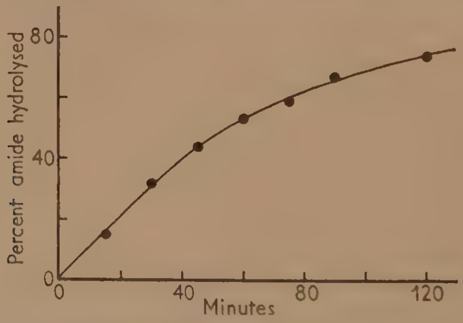

FIG. 1. Showing the relationship between the percentage of γ-MG hydrolysed and reaction time in a typical experiment using a groundnut leaf extract as a source of deamidase. Initial γ-MG concentration, 0·015 M.; reaction pH 7·4.

virtually constant during the first 30 per cent. reaction and then begins to decrease progressively, all rates quoted in later experiments were determined from measurements of the γ-MGA produced during this initial (30 per cent.) reaction period.

Optimum pH of deamidase. The activities of the enzyme using γ-MG and glutamine as substrates were measured at nine pH values within the range 5·6–9·2. The results are shown in Fig. 2, from which it can be seen that the optimum pH for either substrate was about 7·8. The enzyme was fairly rapidly inactivated as the pH was altered away from the optimum, its activity being negligible below pH 5·5 or above pH 9·5. To enable phosphate to retain a high buffering capacity, all further deamidase experiments were performed at pH 7·4, at which the activity of the enzyme was only slightly below its maximum.

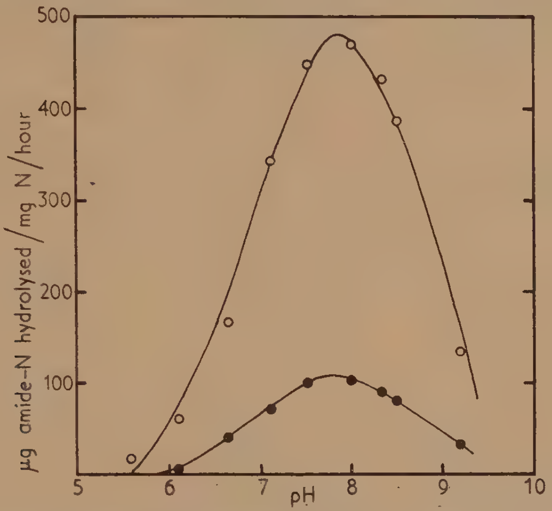

Fig. 2. Showing the relationship between reaction pH and rate of hydrolysis of amino-acid amide by an extract of groundnut leaves. –O—O– γ-MG, –●—●– glutamine.

Specificity of deamidase. The hydrolysing activity of the deamidase was tested for six structurally related amino-acid amides. These were γ-MG, glutamine, asparagine, γ-methyleneisoglutamine, isoglutamine, and γ-methylglutamine. During a 3-hour period no detectable hydrolysis of asparagine, isoglutamine, or γ-methyleneisoglutamine occurred. The reaction rates (expressed in the units defined earlier) for the other amides were: γ-MG, 415; glutamine, 80; γ-methylglutamine, 38. The rate of deamidation of γ-MG remained about five times that of glutamine over the pH range 6·5–9·0 (see Fig. 2).

Effects of various treatments on the deamidase activity. Freeze-drying. Extracts of leaves prepared in the normal way were freeze-dried and stored at −10°. When the residues were redissolved in distilled water 24 or 48 hours later, the full deamidase activities of the original extracts for γ-MG or glutamine were found to have been preserved.

Ammonium sulphate precipitation. Ammonium sulphate was added to portions of an extract of leaves, prior to its dialysis, in amounts that would produce solutions 20, 40, and 60 per cent. saturated with respect to the salt. The precipitated proteins were centrifuged down, and the supernatant liquids discarded. The proteins were then resuspended in a few ml. of pH 7·4 phosphate buffer and dialysed for 4 hours in the normal way. The volumes of the dialysates were then made up to the volumes of the original extracts used, and tested for enzyme activity. Table I compares the activity of each preparation with that originally present in the extract.

TABLE I

Showing the percentages of the total deamidase activity precipitated by various concentrations of ammonium sulphate

$(NH_4)_2SO_4$ concentration expressed as a percentage of saturating concentration	Deamidase activity precipitated as a percentage of initial activity. Substrate γ-MG	Deamidase activity precipitated as a percentage of initial activity. Substrate glutamine
20	10	14
40	71	66
60	97	102

From the table it is seen that the deamidase could be precipitated without loss of activity, and was completely precipitated from solutions which were 60 per cent. saturated with regard to ammonium sulphate.

Storage at pH 5·0. A normal dialysed extract prepared at pH 7·4 was adjusted to pH 5·0 and stored at 0° for 24 hours. It was then readjusted to pH 7·4, and its activity measured and compared with that of the original untreated extract. This storage at the sub-optimal pH did not measurably reduce the deamidase activity of the extract for either γ-MG or glutamine.

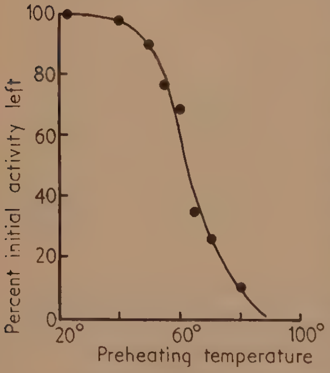

FIG. 3. Showing the effect of a 10-minute preheating period at a series of elevated temperatures on the activity of the deamidase present in an extract of groundnut leaves. Reaction pH 7·4.

Heat treatment. The effect on the deamidase activity of extracts maintained at various elevated temperatures for 10 minutes before use is recorded graphically in Fig. 3. Aliquots of the same extract were preheated at a series of temperatures between 40° and 80°, and the residual activities for γ-MG hydrolysis at the end of the 10-minute heating period were measured and are expressed in Fig. 3 as percentages of the activity of the unheated extract. The loss of deamidase activity towards glutamine was closely similar.

Small volumes of a similar extract were also heated for increasing periods of time at fixed temperatures of 60° and 70°, and the residual deamidase activities again measured and compared with that of the untreated extract. The results, given in Table II, are again expressed as percentages of the initial activity remaining after the heat treatment.

TABLE II

Showing the effect of increased time of heating on the loss of deamidase activity in leaf extracts

Time of heating (minutes)	Percentage of the initial activity remaining after heating at: 60°		70°	
	Substrate γ-MG	Substrate glutamine	Substrate γ-MG	Substrate glutamine
0	100	100	100	100
5	80	80	52	50
10	50	55	24	28
20	25	30	10	13

TABLE III

Showing the deamidase activities of different tissue extracts, and the relative amounts of γ-MG and γ-MGA in these tissues

Tissue	Units of deamidase activity. Substrate γ-MG	Ratio γ-MG/γ-MGA concentrations in tissues
Leaf . . .	386	0·19
Stem . . .	150	3·8
Hypocotyl . .	32	19·2
Root . . .	0	∞

Addition of various ions. To test whether phosphate ions had a specific effect in activating the enzyme, an experiment was performed where tris(hydroxymethyl)aminomethane ('tris') replaced phosphate as the buffer. The leaf extract was prepared in and dialysed against 0·05 M. pH 7·4 tris buffer. The activity of this extract, with and without added phosphate, was then measured. The reaction rate for the tris alone was 155 units with γ-MG as substrate; when 0·02 M. potassium orthophosphate (adjusted to pH 7·4) was present it increased to 410 units, that is an activity of the order encountered in previous experiments where phosphate buffers were used. 0·02 M. potassium pyrophosphate caused an approximate doubling of the rate measured using tris alone, whilst 0·02 M. potassium chloride or cyanide did not measurably alter the rate. The added ions affected the rate of glutamine hydrolysis in a similar manner.

Distribution of deamidase in the organs of groundnut plants. Leaves, stems, hypocotyls, and roots of groundnut plants that had developed to the five-leaf stage were separately harvested, and extracts of each tissue were prepared in the normal way in phosphate buffer. The deamidase activity of these extracts were measured for γ-MG only. The results are given in Table III, together with the γ-MG/γ-MGA concentration ratios determined earlier for these tissues (Fowden, 1954*a*).

A comparison was also made of the distribution of the enzyme in leaves of different ages taken from plants which had developed eight or nine leaves on the main stem. When the leaves on the main stem were numbered counting from the first well-defined leaf (i.e. a higher serial number indicated an older leaf) the following deamidase activities were obtained: leaf No. 1, 210 units; leaf No. 3, 400 units; leaf No. 5, 480 units; leaf No. 7, 500 units.

DISCUSSION

Little detailed information can be found in the literature concerning deamidases present in higher plants. It is therefore necessary to compare the properties of the deamidase in groundnut plants with those found for other deamidases having, in the main, an animal or microbial origin.

Groundnut deamidase has a similar optimum pH to those reported for other deamidase preparations, where pH optima of about 8 are usual for asparaginases (Geddes and Hunter, 1928; Schmalfuss and Mothes, 1930; Errera and Greenstein, 1947; Busch, 1942), and values in the range of 7 to 9 have been reported for glutaminases (Krebs, 1935; Greenstein and Leuthardt, 1948). The optimum pH of the groundnut enzyme is higher than the actual pH of extracts expressed from macerated leaves (normally about pH 6), and so the enzyme is probably acting *in vivo* at the lower end of its pH activity range. This may explain why measurable amounts of γ-MG occur in leaves even though these tissues possess high deamidase activity. If the leaves were to have an internal pH of about 8, it might be expected that complete hydrolysis of any γ-MG which they contained would occur unless some intercellular separation of enzyme and substrate were possible. The pH of the root extracts did not differ significantly from those of leaves taken from the same group of plants, and so the absence of deamidase activity can probably be attributed to the fact that the enzyme is not produced by the roots, rather than to a destruction of the enzyme by low pH values. The second explanation is made more unlikely, since it has been demonstrated that at pH's as low as 5 the activity of the deamidase remains unaffected when it is stored at 0° for 24 hours. This is in marked contrast to the instability of an asparaginase and a glutaminase (Grassmann and Mayr, 1933; Errera and Greenstein, 1947).

Within the group of amino-acid amides tested, the deamidase showed maximum hydrolytic activity towards γ-MG. If the enzyme plays an essential role in the over-all nitrogen metabolism of the groundnut plants, then it would be by controlling the levels of γ-MG occurring within different tissues. The enzyme obviously does not influence the metabolism of the plant by its control on the other physiological amides, asparagine and glutamine, for although the former is present in some tissues in substantial amounts the enzyme is completely inactive towards it, whilst glutamine is only present in the plant at concentrations much lower than those of the other two amides.

The fact that the deamidase activity towards γ-MG is invariably about five times greater than towards glutamine, irrespective of the treatment to which the enzyme has been subjected prior to assaying its activity, strongly indicates that a single enzyme is present in the plant. This is perhaps best regarded as a γ-methyleneglutaminase, which does not show an absolute specificity in substrate requirement. The structural features required in the substrate are, however, fairly exacting, since a four-carbon amino-acid amide, asparagine, or isomers which are structurally closely related to suitable substrates, isoglutamine and γ-methyleneisoglutamine, are not attacked. The enzyme differs markedly in substrate requirement from the glutaminase of *Clostridium welchii* S.R. 12, which does not appear to be capable of hydrolysing γ-MG at all (Fowden, 1954*b*).

The replacement of the $=CH_2$ substituent on the γ-carbon atom in γ-MG by a hydrogen atom and a $-CH_3$ group to give γ-methylglutamine is sufficient to produce a tenfold decrease in the rate of hydrolysis of the amide, whilst even a replacement with two hydrogen atoms (to give glutamine) causes a five-fold fall in the rate. The decreased rate of hydrolysis therefore cannot be attributed to the presence of larger atoms or groups present on the γ-carbon atom causing increased steric hindrance towards enzyme-substrate complex formation, but must be more directly linked to the disappearance of a carbon-carbon double bond from the substrate molecule. The effect produced by substitution of $-CH_3$ for $=CH_2$ in γ-MGA has earlier been reported for two other enzymes. In contrast to the deamidase, the transaminase system present in groundnut plants appears to utilize γ-methylglutamic acid just as readily as γ-MGA (Fowden and Done, 1953*b*). On the other hand, replacement of γ-MGA by γ-methylglutamic acid as the substrate for the decarboxylase of *Clostridium welchii* S.R. 12 is accompanied by an approximately twentyfold fall in rate.

The observed distribution of the deamidase within the different plant organs, and the dependence of the γ-MG and γ-MGA concentrations upon this distribution (see Table III), may constitute an integral part of the over-all nitrogen metabolic scheme. Evidence has been presented (Fowden, 1954*a*) which strongly suggests that in groundnut plants the major portion of nitrogen is translocated from the roots or cotyledons to the developing leaves in the form of γ-MG. At the moment the pathway by which γ-MG itself is synthesized remains unknown, but it may resemble that of glutamine from α-ketoglutaric acid via glutamic acid, since the appropriate keto acid, γ-methylene-α-ketoglutaric acid, has recently been shown to be present in plants which contain γ-MG (e.g. tulips, Towers and Steward, 1954; groundnuts, Fowden and Webb, 1954, 1955). Two enzyme systems, one synthetic and one degradative (the deamidase), then probably control the levels of γ-MG present in any tissue, the large variations in the concentrations of γ-MG and γ-MGA being the result of changes in the deamidase concentrations of different organs, rather than of changes in the concentration of the assumed synthetase. The role of the deamidase in the leaves may be to release, at a controlled rate, half the nitrogen combined in γ-MG as ammonia nitrogen, which would then be utilized for new protein synthesis.

The increase of deamidase activity with increasing age of the leaf which was observed when the activities were expressed in the normal units used requires a further brief comment. Since these activities were related to the total nitrogen contents of the extracts, and not to the dry weights of the leaves initially used, the results merely indicate that the deamidase constitutes an increasingly large proportion of the total protein present in the leaves as they age. But since the total protein content of the leaves decreased markedly during ageing (the total nitrogen content fell from 6·5 to 2·7 per cent. on a dry-weight basis), the absolute concentration of deamidase can be calculated to remain approximately constant, whilst that of the total protein is decreasing to less than half its initial level.

I would like to thank Miss A. E. Wright for technical assistance during the course of this work, and the University of London Central Research Funds Committee for a grant towards the cost of the freeze-drying apparatus used.

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The Translocation of Antibiotics in Higher Plants

I. ISOLATION OF GRISEOFULVIN AND CHLORAMPHENICOL FROM PLANT TISSUE

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SUMMARY

Griseofulvin and chloramphenicol have been recovered from the tissues of broad-bean plants grown in solutions containing these antibiotics.

Partial separation of the compounds from other constituents of solvent extracts of plant tissue was effected by counter-current distribution in a carbon tetrachloride : methanol : water system. Pure griseofulvin and chloramphenicol were then obtained by recovery of the solute in appropriate tubes followed by chromatography on an activated alumina column; they were identified by mixed melting-point determinations and comparison of their infra-red spectra.

A method for estimating griseofulvin in plant tissue extracts based on counter-current distribution and examination of the fractions spectrophotometrically has been developed, and used to strike a balance between the griseofulvin entering the plant and that present in the tissues after treatment.

Estimates of chloramphenicol in the tissues by bioassay agree well with the chemical determinations.

INTRODUCTION

THE literature on the movement of antibiotics in higher plants has recently been reviewed (Crowdy and Pramer, 1955*a*). It has been claimed that these compounds are translocated by the plants but identification has usually rested on the biological assay of the compounds concerned and in no case has the compound actually been recovered from the plant tissue. There must always be an element of doubt in this method of identification. The present communication describes the recovery and chemical identification of two antibiotics, griseofulvin (7-chloro-4 : 6 : 2′-trimethoxy-6′-methylgris-2′-en-3 : 4′-dione, I; R = CH_3) and chloramphenicol, from the tissues of treated broad-bean plants. It has also been possible to estimate the griseofulvin in the tissues spectrophotometrically and compare the weight supplied to the plant with that estimated as present in the plant tissues. The methods used for the estimation of griseofulvin are described in detail. A preliminary account of the isolation

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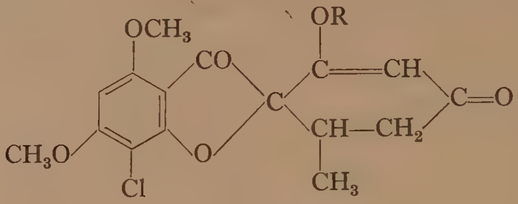
and identification of griseofulvin has already been published (Crowdy, Grove, and Pramer, 1954).



EXPERIMENTAL

Materials. Griseofulvin, m.p. 220–1° C., and chloramphenicol, m.p. 149–50° C., were used.

Plants and preparation of plant extracts. Seed of broad bean (*Vicia faba L.*), var. Sutton's Dwarf, were germinated in vermiculite and the seedlings were transferred to beakers containing the high-nitrogen nutrient solution described by Agarwala (1951). The nutrient was replaced with an aqueous solution of the antibiotics when the 2nd and 3rd leaves were expanding. During treatment the plants were kept in a box at 25° C. and at a relative humidity of about 70 per cent. They were lighted 14 hours daily with high-pressure mercury lights. Treatment was continued for 3 days with griseofulvin and 4 days with chloramphenicol. With *griseofulvin* the plants were either treated in a solution of 25 μg./ml.; or when the intake of the antibiotic was balanced with its recovery from the plants, a known weight of griseofulvin, of the order of 125 mg., was dissolved in 5 l. water and supplied to 25 beakers each containing 4 plants. The roots and shoots were harvested separately and macerated in chloroform in a 'Waring Blendor'. Prior to harvesting the roots were washed in 4 changes of distilled water, a process which had been found to remove practically all the griseofulvin on the surface. In griseofulvin balance experiments the residual griseofulvin in the treating solution and the washing waters was estimated as described below.

The macerates of the roots and shoots were covered with chloroform and allowed to stand in the laboratory for 3 days: extraction was then completed in a Soxhlet apparatus for 2 hours. The chloroform extracts thus obtained were evaporated to dryness. This process yielded about 4·5 mg. of extract per gram fresh weight of shoot tissue and 1·1 mg. per gram fresh weight of root tissue.

With *chloramphenicol* plants were grown in solutions containing 330 μg./ml., roots were discarded, and the shoots macerated in ethyl acetate.

Counter-current distribution of griseofulvin. The detection and estimation of small quantities of griseofulvin directly in the chloroform extract of macerated bean-plant tissue was found to be impracticable and recourse was had to counter-current distribution. Griseofulvin is fairly soluble in methanol but much less soluble in carbon tetrachloride; the green-plant colouring matters, after extraction, are very soluble in carbon tetrachloride and only sparingly soluble in methanol. The solvent system carbon tetrachloride : methanol : water (62 : 35 : 3 v/v) (International Critical Tables, 1928), which readily separated into two phases without emulsification, was selected and was used in the type of apparatus described by Craig (1950), the 50 glass equilibrium tubes each having a capacity for 25 ml. of each phase. The mobile phase was the methanol rich upper phase. The partition ratio (K) of griseofulvin in this solvent was shown to be 2·52. The product (Kv) of the ratio of the volumes of the upper and lower phases (v) and the partition ratio (K) actually operating during the distribution, can be calculated from the equation $N = nKv/(Kv+1)$ where n is the total number of transfers, and N is the number of the equilibrium tube containing the maximum amount of griseofulvin (determined spectrophotometrically). For $n = 50$, N was found to be 33·3 and hence $Kv = 2{\cdot}00$. This value of Kv was then used, in the usual way, to calculate the theoretical distribution curve for 50 transfers (Craig, 1950). This calculation showed that tube 33 should contain a fraction equivalent to 0·118 of the total griseofulvin present; 0·99 of the total griseofulvin was contained in tubes 25–41 inclusive.

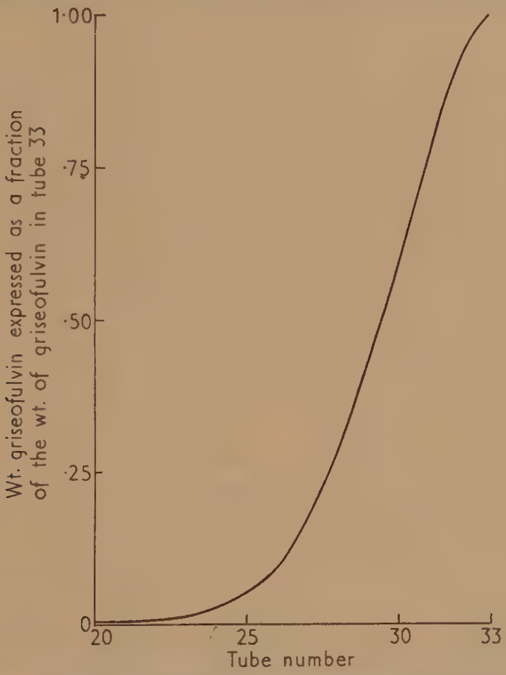


FIG. 1. Counter-current distribution of griseofulvin: theoretical distribution for 50 transfers.

The weight of griseofulvin in each tube from 20–33 expressed as a fraction of the weight in tube 33 is plotted in Fig. 1.

Spectrophotometric estimation of griseofulvin after counter-current distribution. Spectra were determined with a Unicam SP 500 spectrophotometer. It seemed likely from preliminary trials that quantities of griseofulvin of the order of 2–20 mg./g. of plant tissue extract would require estimation giving maximum concentrations in the equilibrium tube upper phases of the order of 3×10^{-5}—3×10^{-4} M. Accordingly it was ascertained that Beer's law held for griseofulvin between these concentration limits, both in ethanol and in the methanol rich upper phase of the carbon tetrachloride : methanol : water system. Griseofulvin shows maxima at 291 and 326 mμ (Grove, MacMillan, Mulholland, and Rogers, 1952): observed molecular extinctions (ε) are as follows:

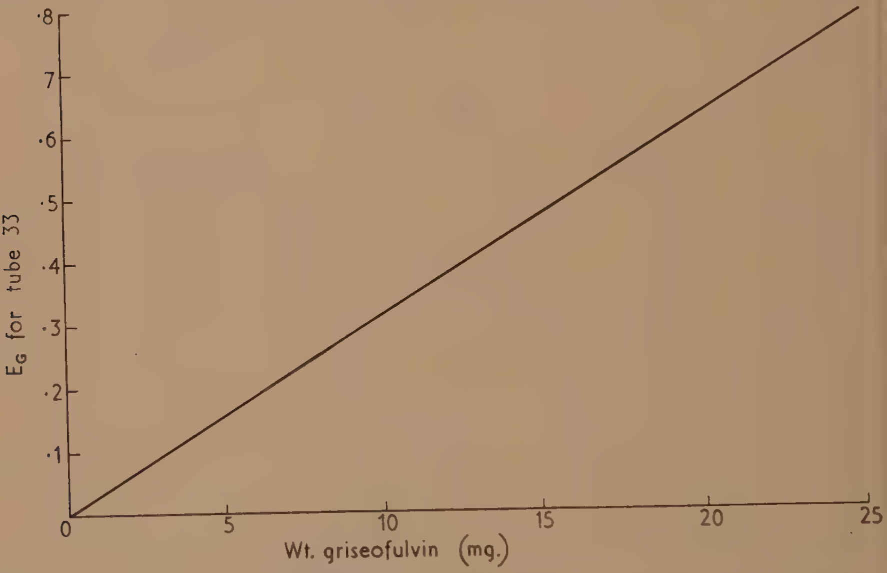


FIG. 2. Spectrophotometric estimation of griseofulvin after counter-current distribution.

Solvent	291 mμ	326 mμ
Ethanol .	24,000	5,000
Upper phase .	23,500	5,000

For most determinations a little of the upper phase was withdrawn from the equilibrium tube, and the extinction ($E = \log._{10}I_0/I$) measured ($l = 0{\cdot}1$ cm.) using upper phase in the solvent cell of the spectrophotometer; the value obtained was multiplied by $(K+1)/K$ ($= 1{\cdot}40$) to correct for griseofulvin in the lower phase giving the extinction (E_G) corresponding to the griseofulvin content of the tube. In more accurate work, including the checking of the calibration graph (Fig. 2), both phases were withdrawn from the equilibrium tube, the solvent was removed, the recovered griseofulvin dissolved in ethanol (25 ml.), and the extinction (E_G) measured. The former method gave results to an accuracy of ±1 mg. which was considered adequate for the work described in this paper. A plot of E_G for tube 33 against the weight of griseofulvin taken for a 50 transfer distribution is given in Fig. 2.

Estimation of griseofulvin in treating solutions. It was found that roots of both treated and untreated bean plants excreted material, which absorbed in the ultra-violet, into the solutions in which they were growing. Consequently it was not possible to estimate griseofulvin directly in the treating solutions by a spectrophotometric method. The interfering substances were, however, almost completely separated from griseofulvin by passage of the recovered mixed solute down a column of activated alumina, and the griseofulvin, after elution, was then estimated spectrophotometrically in the usual way. The analytical method was as follows:

The residue of the solution used for treatment and the washings from the roots were concentrated *in vacuo* at 60° C. from 10 l. to about 2 l. and the concentrate extracted three times with an equal volume of chloroform. The residual solid obtained on evaporation of the dried chloroform extract was dissolved in benzene (20 ml.) and chromatographed in ultra-violet light on a column of alumina (6 × 1·5 cm.) pH 4 activated at 250° C. After coloured impurities had been eluted with benzene (50 ml.), the blue fluorescent (griseofulvin) band was eluted with benzene—1 per cent. methanol (50 ml.). The griseofulvin content of the eluate was then determined spectrophotometrically after removal of the solvent and dissolution of the solute in ethanol (500 ml.). The solute recovered from the ethanol solution was almost pure griseofulvin (m.p. and mixed m.p. 216–17° C.).

When known amounts of griseofulvin of the order of 90 mg. were put through this procedure it was found that while recovery from the alumina column was quantitative within the limits of experimental error, some losses occurred during evaporation of the aqueous solution and its extraction with chloroform. Thus a loss of 10 mg. was recorded during the recovery of 82 mg. griseofulvin from 10 l. water.

Estimation of griseofulvin in plant-tissue extracts. Using pure griseofulvin the counter-current distribution curve for 50 transfers was first determined by spectrophotometric estimation of the extinction at 291 mμ in each tube, and was found to be in good agreement (Fig. 3) with the theoretical curve. A synthetic mixture of griseofulvin in shoot tissue extract (5 mg. g.) was then examined. The green-plant colouring matters remained in the first few tubes but some brown methanol soluble material was present in tubes 39–50 which in addition to absorption in the visible showed high extinctions at 260–80 mμ. The griseofulvin was found between these two batches of tubes, the distribution having been almost completely unaffected by the presence of the plant constituents (Fig. 3). The constituents of the chloroform extract of macerated root tissue similarly had no effect on the griseofulvin distribution curve (Fig. 3).

The constituents of a 1–2 g. sample of shoot extract produced a non-specific light loss or background absorption in the region 250–350 mμ in the griseofulvin containing tubes so that the griseofulvin present could not be measured directly from the value of the extinction at 291 mμ. This difficulty was not encountered with the samples of root extract, which were in any case much smaller (0·2–0·3 g.). When a large sample (5 g.) of shoot extract was subjected to counter-current distribution without prior addition of griseofulvin it was found (*a*) that the background absorption had a constant value at 291 and 326 mμ in all tubes between 25–33 inclusive and (*b*) did not vary from tube to tube within this batch. Above tube 33 the background absorption increased steadily with tube number and with decreasing wavelength due to the presence of increasing amounts of material with $\lambda_{\max.}$ 260–80 mμ. Tubes 27–31 contained small quantities of plant material showing $\lambda_{\max.}$ 285 mμ but the amounts present were too small to cause any appreciable error in estimating griseofulvin, particularly when using the normal sample weight.

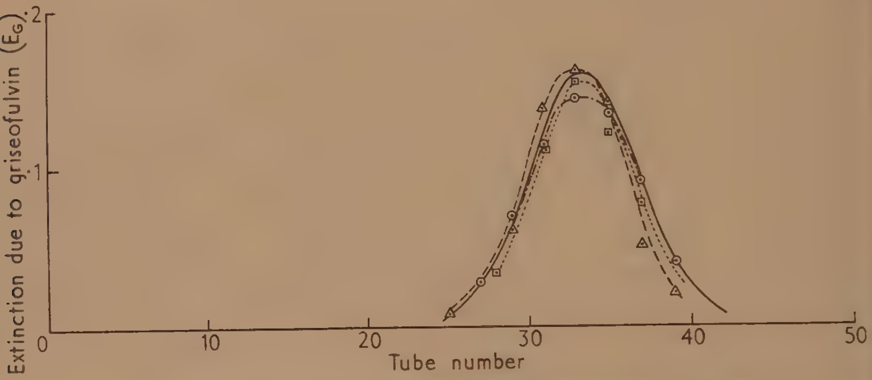


FIG. 3. Counter-current distribution of griseofulvin (5·0 mg.).

———————— Theoretical

– – – – – △ – – – – – Experimental

· — · —⊙— · — · Experimental + shoot extract

⊡·················· Experimental + root extract

In the standardized analytical procedure the extracts of shoots or roots were weighed and transferred with 25 ml. of the lower, carbon tetrachloride rich, phase to tube 0 of the counter-current distribution apparatus and subjected to 50 transfers. The solutions in alternate tubes from 25–39 were then withdrawn and the absorption spectra in the upper phase measured between 250–350 mμ ($l = 0{\cdot}1$ cm.). Two methods were used for estimating griseofulvin in the presence of the background absorption common in a normal sample of shoot extract.

Either (*a*) advantage was taken of the constant background absorption at the two wavelengths 291 and 326 mμ in tube 33 to calculate the griseofulvin content from the difference between the values of the extinctions at these two wavelengths

Thus, let x be the extinction due to griseofulvin at 291 mμ in the upper phase of tube 33

X be the total extinction measured at 291 mμ in the upper phase of tube 33

X' be the total extinction measured at 326 mμ in the upper phase of tube 33.

Then $x = 23500\,(X-X')/18500 = 1{\cdot}27\,(X-X')$

This value of x was corrected for griseofulvin in the lower phase (see above) giving E_G for tube 33. The griseofulvin content of the plant tissue was then read off from the graph (Fig. 2).

Or (*b*) extinctions at 291 mμ in pairs of tubes between 25–33 were measured and the griseofulvin contents of tube 33 calculated by making use of the known theoretical distribution of griseofulvin.

Thus, let

x and X have the same meaning as in (*a*) above,

Z be the extinction due to background absorption at 291 mμ,

Y be the total extinction measured at 291 mμ in the upper phase of tube 27,

Y' be the total extinction measured at 291 mμ in the upper phase of tube 29.

Then from the theoretical distribution (Fig. 1)

$$X = x+Z,$$
$$Y' = 0{\cdot}424x+Z,$$
$$Y = 0{\cdot}170x+Z.$$

Pairs of these equations can be solved for x and Z and the mean value of x taken. This again has to be corrected for griseofulvin in the lower phase giving E_G for tube 33 and the griseofulvin content of the plant tissue obtained from Fig. 2.

Griseofulvin concentrations calculated by both methods (*a*) and (*b*) were usually found to be in good agreement.

Extraction and identification of griseofulvin from shoot tissue and search for griseofulvin metabolites. All griseofulvin derivatives and degradation products which retain the coumaranone ring system intact show ultra-violet absorption similar to griseofulvin with peaks close to 290 and 325 mμ. Before the above analytical methods could be applied to plants treated with solutions containing griseofulvin it was necessary to show that in practice all specific absorption at 291 mμ in tubes 25–41 was due to griseofulvin. At the same time a search was made for possible metabolites of griseofulvin, although it was considered practicable only to search for metabolites showing ultra-violet absorption maxima at wavelengths >250 mμ, i.e. those which still had the aromatic ring or some appropriate system of conjugated chromophores intact.

In a preliminary trial, plants were treated with an aqueous solution containing 25 μg./ml. griseofulvin, and the shoots were extracted with chloroform. The chloroform extract was evaporated and made up to 500 ml. 10 ml. were withdrawn and the two fractions, 10 ml. and 490 ml., of the original solution were evaporated to dryness separately. The smaller sample (C) was prepared for bioassay by a method to be described later (Crowdy, Grove, Hemming, and

Robinson, 1955), and the larger (*B*: 5·5 g.) was subjected to counter-current distribution as described above. Solutions were withdrawn from alternate tubes, and after separation of the phases the upper phase was examined spectrophotometrically ($l = 0{\cdot}1$ cm.) between 250–350 mμ. The results were compared directly with a blank experiment carried out on 5·5 g. (*A*) of chloroform extract of macerated shoots of plants grown for the same time in water without the addition of griseofulvin. The distribution through the apparatus of substances with specific absorption peaks and the corresponding extinctions is set out in Table I.

The griseofulvin content of extract *B* determined spectrophotometrically as above was 12±1 mg.

TABLE I

U.V. absorption of shoot extracts after counter-current distribution

	Extract *A* (blank)				Extract *B* (griseofulvin)			
	Background absorption	Specific absorption Maximum			Background absorption	Specific absorption Maximum		
Tubes	Level (E)	Tube no.	λ(mμ)	(E)	Level (E)	Tube no.	λ(mμ)	(E)
0–10	Green pigments—not investigated				Green pigments—not investigated			
11–14	0·35	13	~280	0·38	0·25	13	~280	0·30
15–24	0·35	21	270–5	0·46	0·25	21	270–5	0·42
25–33	0·40	29	285	0·45	0·30	33	291	0·55
34–38	0·40–0·80	—	—	—	0·35–0·60	—	291	—
39–49	Rising steeply	45	270	~5	Rising steeply	45	270	~5

Tubes 11–14 (peak, tube 13) and *15–24* (peak, tube 21) contained material of $\lambda_{\text{max.}}$ ~280 and 270 mμ respectively but these were plant constituents and present in the blank. *Tubes 39–49* contained large amounts of material of $\lambda_{\text{max.}}$ 260–80 mμ present in the blank; since the quantities present might have been sufficient to mask small amounts of griseofulvin metabolites, the brown resinous solute from these tubes was bulked (0·6 g.), and extracted with *n*-butanol (50 ml.); insoluble material (0·1 g.) was rejected. The butanol solution was extracted in turn with (*a*) sodium hydrogen carbonate (2×25 ml.) and (*b*) 2N hydrochloric acid (2×25 ml.). The basic and ether-soluble acidic fractions, on recovery, showed only background absorption in the ultraviolet. The water-soluble acid fraction showed a sharp peak at 265 mμ present in the blank. The neutral fraction was recovered and chromatographed on alumina using the fractional elution technique. A number of fractions were eluted which, on recovery and dissolution in ethanol, had specific ultra-violet absorption between 265–80 mμ; all these fractions were plant constituents and were present in similar amounts in the blank experiment as judged by a comparison of the extinctions.

The contents of *tubes 27–38*, containing 11±1 mg. griseofulvin by spectrophotometric estimation, were withdrawn, the solvent removed, and the solute dissolved in ether (20 ml.). The ether was extracted in turn with (*a*) sodium hydrogen carbonate and (*b*) 2N hydrochloric acid. Recovery of the acidic and basic fractions in the usual way showed the absence of components with specific ultra-violet absorption. The neutral fraction was recovered, dissolved in benzene, and chromatographed on a column (6 × 1·5 cm.) of activated alumina in ultra-violet light. After a little brown colouring matter had been eluted with benzene, the blue fluorescent griseofulvin band was eluted with benzene-methanol. Details are given in Table II.

TABLE II

Chromatography of shoot extract B: Tubes 27–38, neutral fraction

Eluate	Band	Product	U.V. absorption
1. Benzene (25 ml.)	—	Dark coloured impurities	Non-specific
2. Benzene + 1% methanol (100 ml.)	Blue fluorescent band	Green glass	$\lambda_{max.}$ 291 mμ.
3. Benzene + 1% methanol (100 ml.)	Blue fluorescent band	Nothing further eluted	

The green glass from eluate 2 was sublimed at 180–200° C./10^{-1} mm.; the orange paste-like sublimate was dissolved in hot benzene (0.2 ml.) and light petroleum, b.p. 60–80° C. (1·5 ml.) added. The greenish coloured crystals, m.p. 180–210° C., which separated on standing, crystallized from ethanol in colourless prisms (7·0 mg.), m.p. and mixed m.p. with griseofulvin 218–20° C. The infra-red spectrum of the recovered material was identical with that of griseofulvin.

A blank experiment with 10 mg. griseofulvin showed that 3 mg. were lost during this purification procedure mainly in the sublimation and crystallization stages: recovery from the alumina column was almost quantitative.

Allowing for losses therefore, the weight of pure griseofulvin recovered is in good agreement with the weight believed to be present from the spectrophotometric estimation.

Extraction and identification of griseofulvin from root tissue. The chloroform extract (0·3 g., *E*) of macerated root tissue of plants grown for 3 days on a solution containing 25 mg./ml. griseofulvin was treated as described for the shoot tissue extract. A blank experiment (0·3 g., *D*) on roots of plants grown for the same time in water was run in parallel and the results are contained in Table III.

The griseofulvin content of extract *E* measured in the usual way was 14±1 mg. Tubes *40–47* contained a little material $\lambda_{max.}$ 270–75 mμ present in the blank, but no other material with specific ultra-violet absorption was found. The contents of tubes *27–39* were worked up as described for the shoot extract. No acidic or basic components with $\lambda_{max.}$ 291 mμ were found and the material with $\lambda_{max.}$ 291 mμ in the neutral fraction consisted entirely of griseofulvin (9.8 mg.), m.p. and mixed m.p. 218–20° C. Allowing for losses during purification, the weight of griseofulvin recovered is again in good agreement with the spectroscopic estimation.

TABLE III

U.V. absorption of root extracts after counter-current distribution

	Extract *D* (blank)				Extract *E* (griseofulvin)			
	Background absorption	Specific absorption Maximum			Background absorption	Specific absorption Maximum		
Tubes	Level (E)	Tube no	λ(mμ)	(E)	Level (E)	Tube no.	λ(mμ)	(E)
0–10	Not investigated				Not investigated			
11–26	0	—	—	—	0	—	—	—
27–39	0	—	—	—	0	33	291	0·33
40–47	0·05	44	270–5	0·09	0·05	46	270–5	0·12
48–50	0	—	—	—	0	—	—	—

It is concluded that the specific absorption at 291 mμ in the counter-current distribution of both shoot and root extracts is due solely to griseofulvin and not to any griseofulvin metabolite, either neutral, basic, or acidic.

Translocation of griseofulvin: balance sheet after 3 days. The analytical methods, whose validity was established in the preceding sections, were now applied to a typical 3-day uptake experiment and the balance sheet drawn up (Table IV).

TABLE IV

Uptake and distribution of griseofulvin

	Griseofulvin	
Location after 3 days	(mg.)	% total
Shoots	11	8·9
Roots	20	16·3
Remaining in solution*	82	66·7
	113	91·9
Total taken	123	—
Unaccounted for (metabolized?)	10	8·1

* Corrected for losses during recovery (see above).

Of the griseofulvin taken up by the plants (33·3 per cent. of that presented) about 49 per cent. was present in the roots, 27 per cent. in the shoots, and 24 per cent. unaccounted for.

The decay of griseofulvin in the tissues of the bean will be considered in a later publication (Crowdy *et al.*, 1955). However, ther ates of decay recorded in the latter work are of the correct order to account for the 24 per cent. unaccounted for in Table IV.

Extraction of chloramphenicol from shoot tissue. The solvent system, used to separate griseofulvin from plant constituents, was not so satisfactory when applied to chloramphenicol, where ethyl acetate was used to extract the macerated shoots. Ethyl acetate extracts of plant tissue contained a greater proportion of brown components with high partition coefficients than had been noted in the griseofulvin experiments where chloroform had been employed. Chloramphenicol also has a high partition coefficient (K = *c.* 6) in this solvent system and although separation from the carbon tetrachloride soluble green plant constituents was complete after 50 transfers, tubes 38–48, where it was expected that chloramphenicol would be found, were heavily contaminated with brown components. Moreover, these components showed absorption in the ultra-violet at 260–80 mμ, which made detection of chloramphenicol ($\lambda_{\text{max.}}$ 275 mμ) difficult and quantitative estimation impossible at this stage. However, this brown material was not adsorbed from a benzene solution by a column of activated alumina; chloramphenicol was strongly adsorbed but could be recovered by elution with benzene-methanol and a clean separation was achieved in this way. The isolation and quantitative estimation of chloramphenicol was achieved as follows:

The ethyl acetate macerate from 360 g. fresh weight shoot tissue was twice washed with fresh ethyl acetate and the combined solvent extracts dried over sodium sulphate and taken to dryness at 40–50° C. under reduced pressure. The shoot tissue contained 35 μg./g. fresh weight chloramphenicol by bioassay (Pramer, 1954), equivalent to 13 mg. chloramphenicol.

The dried ethyl acetate extract (1·5 g.) was subjected to 50 transfers in the counter-current distribution apparatus. The contents of tubes 38–48, which all contained material with $\lambda_{\text{max.}}$ 270–80 mμ, were bulked and the solvents removed; the residual brown tar was extracted with hot benzene (30 ml.) giving an insoluble brown residue (*A*), whose presence was largely attributed to the solvent used for extraction, and a dark brown filtrate (*B*). These were examined separately.

(*B*) was chromatographed in ultra-violet light on a column (6×1·5 cm.) of alumina pH 4 activated at 250° C. The dark-coloured impurities were not adsorbed by the alumina and passed rapidly through the column and elution with benzene was continued until their removal was complete. Fluorescent bands were then eluted with benzene-methanol. Fractions were recovered and their ultra-violet adsorptions ($l = 0{\cdot}1$ cm.) measured in ethanol solution (20 ml.).

Eluate I Benzene, 50 ml. A brown tar, rejected.
,, II Benzene+1 per cent. methanol, 200 ml. Light green fluorescent band with orange fringe giving a yellow gum (10 mg.) containing a little material $\lambda_{\text{max.}}$ 275 mμ ($\not>$ 2 mg. chloramphenicol).
,, III Benzene+2 per cent. methanol, 200 ml. Darker green fluorescent band giving a yellow gum (25 mg.) $\lambda_{\text{max.}}$275 mμ; $E = 1{\cdot}75 \equiv 11$ mg. chloramphenicol.
,, IV Benzene+2 per cent. methanol, 300 ml. Colourless eluate giving only a trace of oil. Non-specific ultra-violet absorption.

The gum from Eluate III was extracted with hot benzene (8 ml.) and insoluble material rejected. On concentration to 1 ml., small greyish green prisms, m.p. 145–6° C. (7.0 mg.), separated and were purified by sublimation at 140–5° C./10^{-1} mm. followed by crystallization from water or benzene. The product formed colourless needles of chloramphenicol, m.p. and mixed m.p. with an authentic specimen, 149° C. Identity was confirmed by comparison of the infra-red absorption spectra.

The gum from Eluate II, when treated similarly, yielded a further small quantity (0·8 mg.) of chloramphenicol.

The brown residue (*A*) contained much inorganic material as judged by the residue left on combustion of a small portion. It was suspended in sodium hydrogen carbonate (10 ml.) and a little insoluble resinous material which had no ultra-violet absorption rejected. On acidification and extraction with ether a little brown gum was obtained ($\lambda_{\text{max.}}$ 275 mμ), but this on treatment with benzene as described above for (*B*) Eluate III yielded a solid (0·5 mg.), m.p. 130–200° C. (dec.), which was not chloramphenicol and was in insufficient quantity for further work.

The aqueous solution remaining after ether extraction showed $\lambda_{\text{max.}}$ 260–5 mμ and was considered free from chloramphenicol.

The weight of chloramphenicol recovered was in good agreement with that calculated to be present both from the bioassay and from spectrophotometric examination of Eluate III from the alumina column. The bioassay therefore provides a reliable estimation of the chloramphenicol present in shoot tissue.

DISCUSSION

The work described above shows that counter-current distribution, employing a suitable solvent system, coupled with chromatography on activated alumina can effect almost quantitative separation of both griseofulvin and chloramphenicol from the tissues of bean plants. The weights of griseofulvin isolated are in satisfactory agreement with the weights estimated spectrophotometrically after counter-current distribution. The spectrophotometric method can, therefore, be used for the determination of griseofulvin in tissues extracts. The weight of chloramphenicol isolated from shoot tissue is also in good agreement with the weight estimated spectrophotometrically after counter-current distribution and chromatography.

In the case of chloramphenicol it is shown that bioassay provides a reliable estimate of the actual quantity of the antibiotic present in the shoots. In a later paper (Crowdy *et al.*, 1955) it will be shown that the bioassay for griseofulvin in bean shoots is also reliable.

The experiments demonstrate clearly that both griseofulvin and chloramphenicol are taken up by bean roots and translocated to the shoots unchanged. It has been suggested by Crowdy and Pramer (1955*b*) that griseofulvin exists in the tissues in a free form which is expressed in the cell sap, and a bound form which may only be extracted with organic solvents. It can now be said with confidence that the formation of covalent linkages between griseofulvin and plant constituents is not involved.

In the experiments in which griseofulvin was isolated from plant tissues, no metabolites of griseofulvin could be detected elsewhere in the apparatus as a result of the counter-current distribution. In particular no 7-chloro-4 : 6-dimethoxy-2′-methylgrisan-3 : 4′ : 6′-trione (I; R = H, enolic form), the acid hydrolysis product of griseofulvin (Grove *et. al.*, 1952), was detected. A slow break down of griseofulvin in the tissues is known to take place

(Crowdy *et al.*, 1955) but the duration of this trial was evidently too short to allow the detection of metabolites. This last conclusion was confirmed by the balance sheet drawn up for the 3-day uptake experiment, in which only 10 mg. of the griseofulvin presented to the plants could not be accounted for.

We thank Mr. P. W. Jeffs and Misses A. Petrie and K. C. Robinson for assistance, and Dr. L. A. Duncanson for the infra-red spectra.

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Freezing of Plant Tissues under the Microscope

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WITH 3 PLATES

SUMMARY

The freezing of plant cells under the microscope was studied on three types of tissues: *Tradescantia* staminal hairs, fruit skin, and moss leaf. Cinemicrography was used to record stages in the freezing processes, which are otherwise unobservable. The following phenomena have been demonstrated and discussed: (1) The protective effect of persistent supercooling. (2) Ice-inoculation of cells and factors affecting it. (3) Sequence of ice formation within cells. (4) Modes of ice formation as affected by supercooling. (5) Freezing of cell walls and their longitudinal splitting.

THE microscopic study of changes taking place during freezing of plant cells allows some insight into the fundamental processes of frost damage. Such observations were made by Molisch (1897) and later by several others; Matruchot and Molliard (1902), Chambers and Hale (1932), Siminovitch and Scarth (1938), Stuckey and Curtis (1938), Genevès (1954), Luyet (1954), Modlibowska and Rogers (1954).

In this last and in the present study cinematography was used in view of the high speed at which the freezing processes take place. This made it possible to obtain a continuous record of these changes and to study in detail certain important stages which otherwise remain unobservable, e.g. the actual ice inoculation of a cell.

MATERIAL AND METHODS

Three types of plant tissue were investigated. (1) Chains of large cells from a plant hair (*Tradescantia* sp. staminal hairs). (2) Coloured fruit skin (sweet cherry and *Berberis* sp.). (3) A single layer of large cells of a moss leaf (*Pterygophyllum lucens*).

As the criterion of life in *Tradescantia* cells cyclosis was accepted. In the coloured fruit-skin cells the loss of pigment was a certain sign of death. In dead cells of a moss leaf the degeneration of the chloroplasts was obvious.

The tissue was mounted in a drop of water (unless otherwise stated) on a perspex slide fitted with a 40 s.w.g. copper-constantan thermocouple. The cold junction was kept at 0 °C. in ice and water. The electric potential produced by the thermocouple was amplified by a Sunvic amplifier and recorded continuously by a modified Kent recorder.

Journ. of Experimental Botany, Vol. 6, No. 18, pp. 384-91, October 1955.

The tissue was cooled by placing a piece of solid carbon dioxide on one side of the slide covered with a copper plate to aid heat conduction. Condensation on the slide and lenses was overcome by application of glycerol to the surfaces.

The microscope was fitted with a camera or alternatively with a cinecamera. A viewing eyepiece, incorporating a semi-reflector, allowed the process of freezing to be followed and photographed or filmed during the periods of greatest interest. Many of the photographs in this paper are stills from the films.

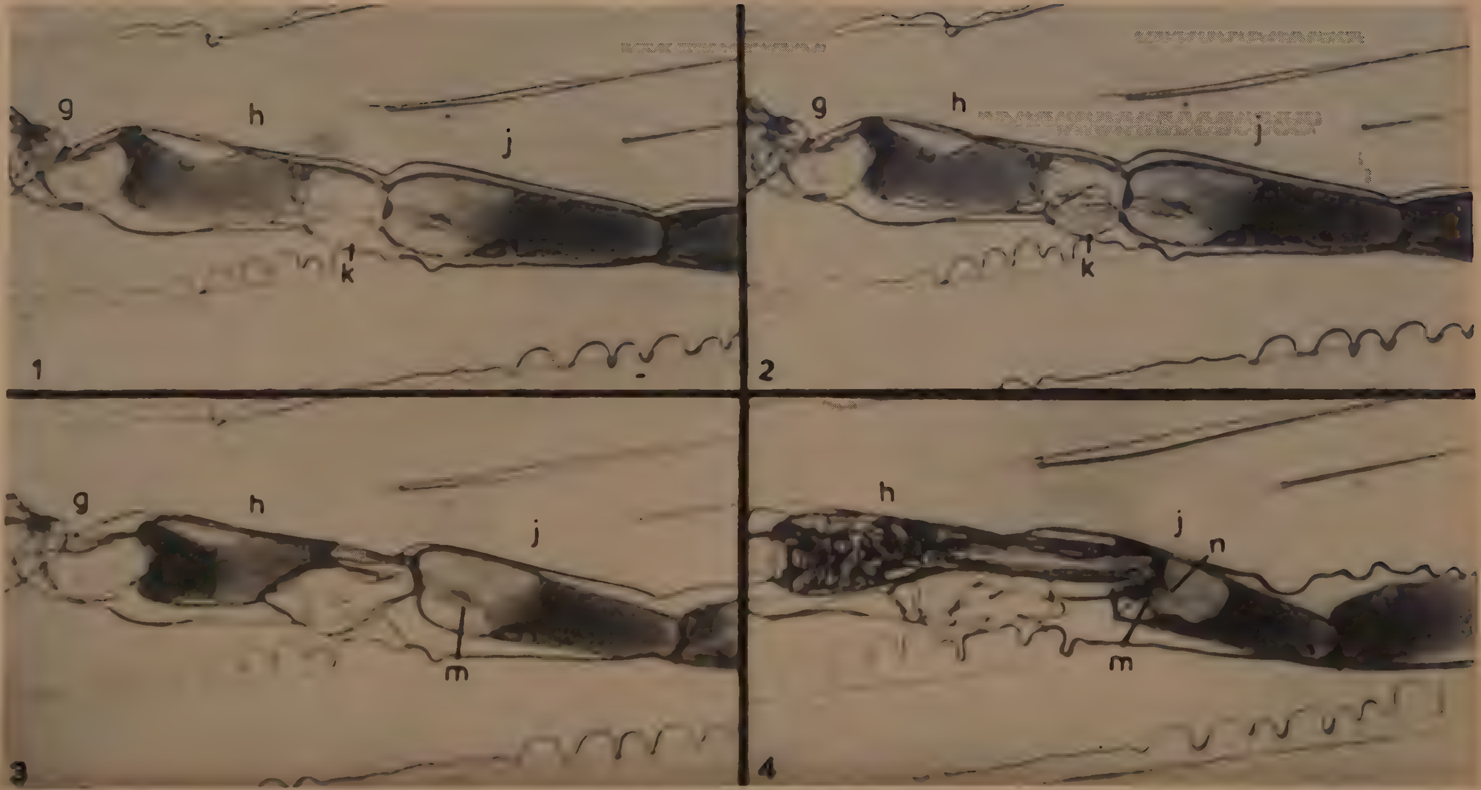
Supercooling. The conditions on the microscope slide are characteristic of a capillary and greatly favour supercooling (lack of crystallization at temperatures below the freezing-point). To overcome it, the medium was seeded by contact with the solid carbon dioxide. Fig. 5 shows the spicules of ice, resulting from such inoculation, invading the medium in which two *Tradescantia* hairs are mounted. Two cells of the lower hair are frozen.

Living undamaged cells have a strong tendency to supercool. Those cells which supercool persistently throughout the exposure to temperatures below their freezing-point survive, while similar cells in the same temperature conditions, but with ice formed inside them, are killed. Thus it is not the temperature but the intracellular ice formation that kills the cells. This is clearly illustrated by Figs 6 and 7.

Fig. 6 shows two *Tradescantia* hairs from the same stamen mounted in opposite directions (i.e. head to tail) in a drop of water. The preparation has been cooled and inoculated from the left side, and ice surrounds the cells in the field of view. These are in contact and thus as nearly as possible under identical conditions. Yet only the lower hair is freezing, while the upper one remains supercooled. This different behaviour is due to the ice inoculation of the lower hair, facilitated by the wounded basal cell, and to the lack of ice inoculation of the undamaged tip cells of the upper hair. If the cooling had been continued until the ice crystals had reached and inoculated the basal cells of the upper hair, it also would have frozen; but the preparation was warmed again before this happened. Thus, the behaviour of the lower hair is an example of 'temporary supercooling' (supercooling followed by freezing); and that of the upper hair, of 'persistent supercooling' (supercooling throughout the exposure to low temperature).

In Fig. 6 ice (white) is lining the walls and surrounding the nucleus of cell (*a*). The vacuole (dark) in the centre of cell (*a*) has shrunk as a result of the dehydrating action of the thickening ice lining. Cell (*b*) is just being inoculated from cell (*a*) with small ice crystals appearing black on the dark background of the vacuole.

Fig. 7 shows the same cells as Fig. 6 one hour after thawing. The cells of the lower frozen hair were killed and are now in an advanced stage of degeneration. Their protoplasm is coagulated, ruptured, and torn away from the cell walls. These dead cells swell, increasing in width. The hair cuticle has separated in some places from the cell wall and this space (*d*) has become filled with the pigmented cell sap which has escaped from the disintegrated



FIGS. 1–4. Plasmolysed *Tradescantia* cells in process of freezing.

FIG. 1. Cells (*g*, *h*, *j*) surrounded with ice, (*k*) lifted cuticle. FIG. 2. Ice inoculation across the lifted cuticle (*k*). FIG. 3. Ice inoculation from cell (*g*) into the vacuole of cell (*h*), (*m*) protoplasmic strands. FIG. 4. Freezing of cell (*h*) and ice inoculation of cell (*j*); (*m*) protoplasmic strands, (*n*) ice crystals inoculating cell (*j*). (magnification, approx. × 105.)

vacuole. The cells of the upper, supercooled, hair survived the cold treatment unharmed. After one hour at room temperature they also imbibed some water but swelled more evenly in all directions. The cell (*c*) also shows some lifting of the cuticle from the cell wall (*e*). In contrast, however, to the killed cell (*a*) the space (*e*) between the cuticle and the cell (*c*) remained colourless indicating that the pigmented cell sap is still firmly held in the undamaged vacuole. Separation of the cuticle in *Tradescantia* hairs usually occurs in old flowers. It is favoured by high temperature which facilitates the hydrolysing processes causing the separation of both walls. Osmosis may then stimulate the lifting of the cuticle.

Ice inoculation. The supercooled state of a plant cell is most commonly ended by ice inoculation; if the cell is protected from ice inoculation, supercooling may persist. The cuticle, the cell wall, and the ectoplasm together form an effective barrier: damage to them facilitates ice inoculation, and thus wounded cells always freeze first. These in turn inoculate other cells across the non-cutinized cell walls.

Some of these points are illustrated by Figs. 1 to 4 which show the freezing of plasmolysed cells of a *Tradescantia* hair. In Fig. 1, separation of the cuticle from the cell wall is seen at (*k*). This facilitated ice inoculation across the cuticle alone (Fig. 2, *k*), but the cells (*h*) and (*j*) themselves remained unfrozen. The freezing bulge enlarged rapidly at the expense of cell (*h*) from which it withdrew water (Fig. 3) and thus did not inoculate it with ice. Cell (*h*) became inoculated with ice from the previously frozen cell (*g*). Ice crystals (appearing black) may be seen invading the vacuole (grey) from the left (Fig. 3). This suggests that the cross walls between cells are more easily penetrated by ice, possibly through the pitfields and along plasmodesmata, than are the lateral walls.

The mode of inoculation of cell (*h*), not through the wall beneath the freezing bulge, but from the end of the previously frozen cell, illustrates the action of another factor, permeability to water.

Plasmolysed cells show a very much higher permeability to water than similar unplasmolysed cells (Myers, 1951). This is probably due to the separation of the ectoplasm from the more permeable cell wall, which also causes the ectoplasm to stretch (Fig. 4, *h*). When a cell possesses the ability to lose water at a rate which allows the cell sap concentration to increase, and thus its freezing-point to decrease, at a rate comparable to the rate of fall in temperature, ice may be formed only extracellularly and the cell may survive. Otherwise intracellular freezing would take place killing the cells. This is clearly demonstrated in Figs. 3 and 4.

The formation of ice in the vacuole of the cell (*h*) caused it to fragment and vesiculate (Fig. 4) and sharply raised its sap concentration. As a result the freezing cell (*h*) rapidly withdrew water from cell (*j*) not yet frozen. Cell (*h*) elongated considerably (over 40 per cent.) and pushed into the end of cell (*j*) (Fig. 4). In this case the moment of ice inoculation of the cell (*j*) depended not only upon the water permeability of the cell walls and plasma membranes, but also on their elasticity and extensibility. By the expansion of the cell (*h*) its wall pressure greatly increased and hampered the water inflow from the cell (*j*) to such an extent that ice from the cell (*h*) inoculated the cell (*j*). In this case ice crystals formed first in the extravacuolar (light) space in the form of dark streaks (Fig. 4, *n*). They should not be confused with the shrunken protoplasmic strands appearing as black threads (Fig. 4, *m*). This is in clear contrast to the intravacuolar ice formation in the cell (*h*) (Fig. 3). It shows that the sequence of ice formation in a plasmolysed cell depends greatly upon the region of ice inoculation.

While in the *Tradescantia* staminal hairs the sequence of freezing cells one after another is obvious, this is not so in the fruit skin. Here the pattern of frozen cells is scattered (dark cells in Fig. 8); but the factors causing it are the same in both cases, viz.: An undamaged cuticle protects both the hair and the epidermal cells from inoculation; and ice inoculation takes place primarily through wounded cells, which, in the case of the skin stripped off a cherry fruit, are on the underside. This point is even better illustrated by the *Berberis* fruit skin (Fig. 9). The epidermal cells are small and thick walled. Those underneath are two to three times larger and have thin walls. They are liable to mechanical damage when the skin is stripped, and thus they are the first to freeze (Fig. 9) and in turn inoculate the overlying epidermal cells.

Modes of intracellular ice formation. The mode of ice formation largely depends upon the degree of supercooling. Ice within a cell may form either (1) *slowly* from a few inoculating ice crystals, which grow by accretion spreading inside the cell and giving it a light appearance (Fig. 10) or (2) *instantaneously* from the many centres of crystallization formed during supercooling. The numerous ice crystals scatter the light and make the cell appear dark. Fig. 11 shows the light cells of a slowly frozen hair of *Tradescantia* on the left and the dark cells of a supercooled and instantaneously frozen hair on the right.

The contrast between cells frozen in these two ways is also clearly illustrated by Fig. 12. The cells of the moss leaf (*Pterygophyllum lucens*) were of similar size before freezing. The cells which froze first without much supercooling are the large, light-coloured ones. While freezing they withdrew water from the cells that were still supercooled, greatly swelling at their expense. The dark, narrow cells are those which froze later, following supercooling.

Extracellular ice formation. Intracellular freezing usually kills the cells, while the more common extracellular freezing, i.e. in the intercellular spaces, although fatal in some cases, may in others exercise even a protective effect (Modlibowska, 1951).

Ice usually forms first in the intercellular spaces. In the moss leaf, which has no such spaces, ice was observed forming first in the cell walls themselves and was then followed by intracellular freezing. Cell walls may, however, crack longitudinally while freezing. The actual process of such splitting has been observed under the microscope and recorded on the film. Ice, forming presumably in the region of the middle lamella, separated the cells without breaking their walls and produced a cavity (Fig. 13, *a*). This cavity enlarged by the freezing of water which was withdrawn not only from the adjacent cells but also from those further away so long as they were unfrozen. During thawing the cells reimbibed the lost water and the cavity closed.

Longitudinal splitting of cell walls by ice frequently occurs under natural conditions in leaves and flowers (Modlibowska, 1951). Fig. 14 shows the skin (epidermis and 3-cell-thick hypodermis) of the receptacle of an apple flower, lifted by ice a few days before this photograph was taken. This split between cells of the cortex and the hypodermis occurred in a similar way to that observed in the moss leaf under the microscope. Ice formed between the cell walls, withdrew water from the surrounding tissues several cells deep, and thus protected them from freezing. After thawing, the separated but otherwise undamaged cells began dividing and forming the large callus cells (Fig. 14, *d*) which are filling and healing the wound.

DISCUSSION

This work has confirmed and demonstrated that it is not the low temperature itself, but the intracellular ice formation which is directly responsible for the frost killing of plant cells.

Cells may avoid damage by frost in two main ways: (1) by resistance to ice inoculation from *outside*, the cell thus remaining supercooled, (2) by resistance to ice formation *inside*, e.g. by maintaining a cell content concentration that will not freeze at a given temperature. Usually both these protective mechanisms are jointly responsible for the survival of plant tissues at low temperature.

Ice inoculation may be prevented by the low water content of the cell wall and its structural resistance to ice penetration, e.g. cutinization, waxiness, and other protective coverings of whole organs.

Resistance to intracellular ice formation is determined by the physical and chemical properties of the whole cell. A low water content and/or an ability to lose water rapidly play a vital role. High water permeability of the ectoplasm is particularly important, as it enables the cell to lower the freezing-point of its sap in pace with the fall of the ambient temperature.

This microscopical study of freezing tissues has shown the important effects not only of 'persistent' supercooling but also of extracellular ice formation within the tissues. In tissues devoid of intercellular spaces, e.g. in the moss leaf, the actual freezing of cell walls as a separate process preceding the intracellular freezing was frequently observed. This in some cases resulted in the longitudinal splitting of the cell walls along the middle lamella, leaving the separated cells undamaged.

Some conclusions may be drawn as to the sequence of freezing in plant cells. It has been shown that although by natural inoculation some ice must form first in the protoplasm before it reaches the vacuole it need not necessarily spread first in the extravacuolar part of the cell. As a result of special conditions, e.g. in the plasmolysed cell (Fig. 6) the freezing may begin first in the vacuole. The usual sequence of slow freezing following ice inoculation is, however, as shown in Fig. 2, formation of ice along the cell wall fromwater withdrawn from the protoplasm and the vacuole, which consequently contracts. This is followed by freezing of the vacuole.

In cells frozen following a temporary supercooling, the numerous crystals, appearing instantaneously, obscure the view and it is difficult to determine their origin. It seems likely, however, that in deeply supercooled cells freezing spontaneously nuclei of crystallization may form first in the vacuole, where the water molecules might be expected to reorientate more freely than in the other parts of the cell.

The widened knowledge of the behaviour of cells during freezing, which these cinemicrographic studies have given, suggests new possibilities for future work.

For instance, since wounds play a large part in providing a channel for ice inoculation, work on the effects of minute wounds caused by insects or by spray damage, either mechanical or chemical, may be worth while.

Further the present studies give new support to the idea that certain substances might be applied to modify the reaction of the plant cells to frost. Such substances might plasmolyse the cells and raise their sap concentration and the permeability of their membranes, or might, given adequate penetration, retard crystallization and reduce the amount of ice formed. The microscopic method should provide a rapid means of testing possible substances and of obtaining indications of the types of substance that will give favourable results.

ACKNOWLEDGEMENTS

The authors thank Mr. C. H. W. Slater for his valuable help with the instruments, Mr. N. MacQueen for taking the film, and Miss L. I. Scott for supplying the moss, *Pterygophyllum lucens*.

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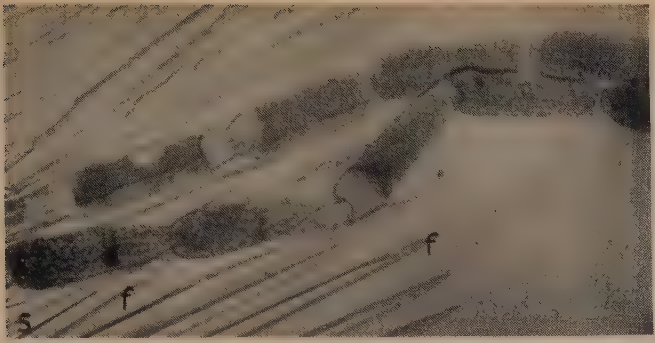
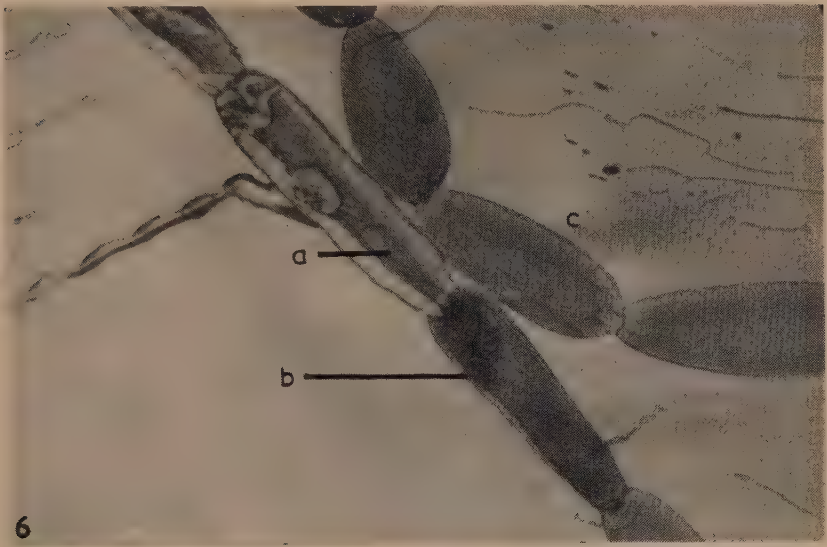
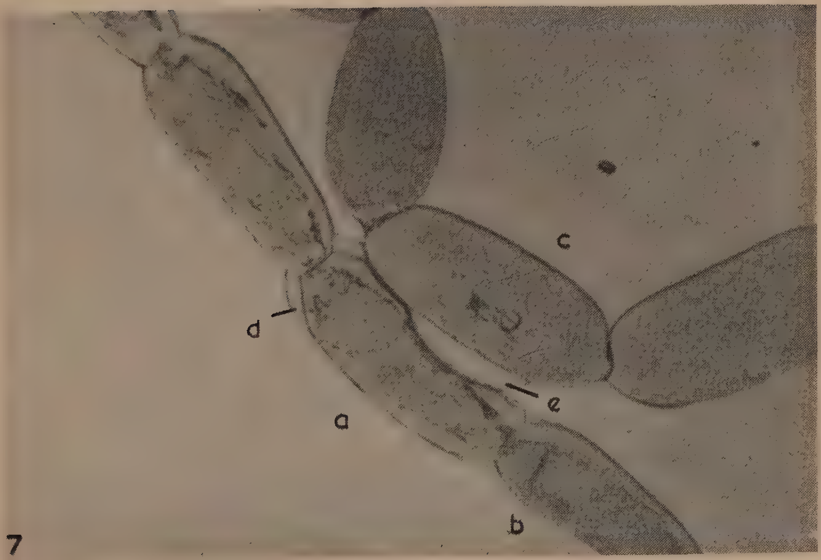
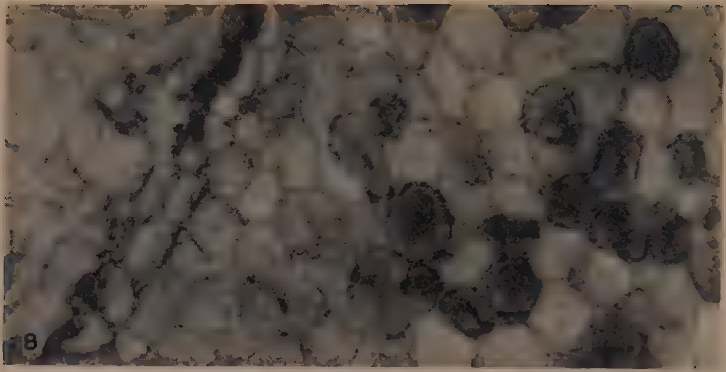






I. MODLIBOWSKA *and* W. S. ROGERS—PLATE I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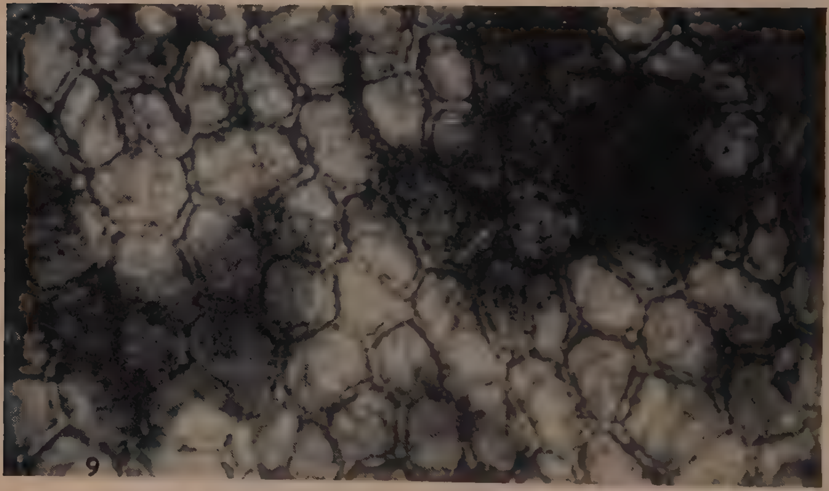
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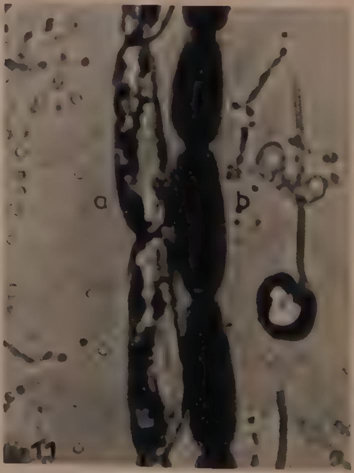
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I. MODLIBOWSKA *and* W. S. ROGERS—PLATE II
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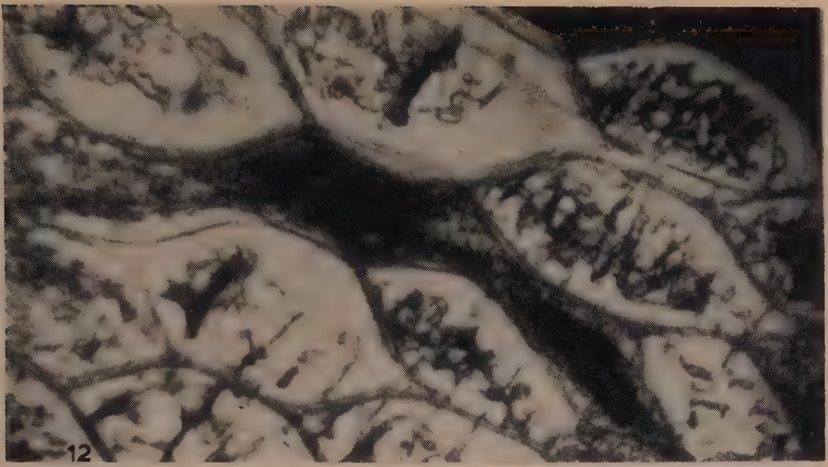

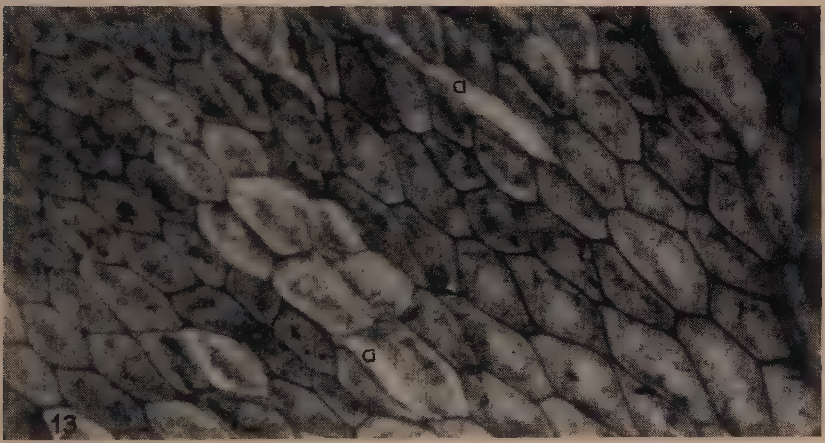

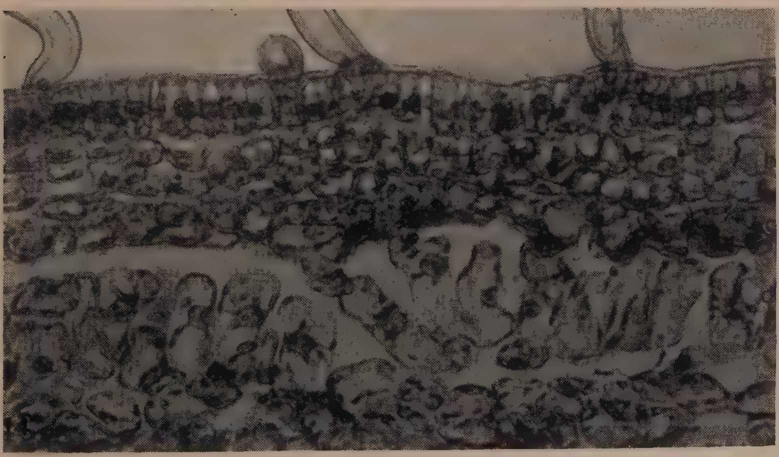

I. MODLIBOWSKA *and* W. S. ROGERS—PLATE III
See description of plates)

DESCRIPTION OF PLATES

PLATE I

FIG. 5. Freezing of *Tradescantia* staminal hairs: *f—f* = frozen cells. (magnification, approx. × 90.)

FIG. 6. *Tradescantia* staminal hairs, freezing (lower), and supercooling (upper): (*a*) freezing cell, (*b*) ice inoculated cell, (*c*) supercooled cells. (magnification, approx. × 110.)

FIG. 7. The same cells as in Fig. 6, one hour after thawing: (*a*) and (*b*) frozen cells, (*c*) cells which supercooled, (*d*) and (*e*) lifted cuticle. (magnification, approx. × 110.)

PLATE II

FIG. 8. Freezing cells of cherry epidermis. The frozen (black) cells form a scattered pattern, due to inoculation from the damaged underside. (magnification, approx. × 500.)

FIG. 9. Freezing of skin of *Berberis*. The large hypodermal cells which have already frozen show up black beneath the smaller (thick walled) epidermal cells. (magnification, approx. × 1000.)

FIG. 10. Ice slowly spreading in a *Tradescantia* hair cell. (magnification, approx. × 100.)

FIG. 11. *Tradescantia* staminal hairs: (*a*) slowly frozen, (*b*) instantaneously frozen following temporary supercooling. (magnification, approx, × 100.)

PLATE III

FIG. 12. Cells of a moss leaf (*Pterygophyllum lucens*). Slowly frozen cells appear light, and instantaneously frozen ones dark. (magnification, approx. × 400.)

FIG. 13. Longitudinal splitting of cell walls in the moss leaf (*Pterygophyllum lucens*): (*a*) cavities filled with ice. (magnification, approx. × 150.)

FIG. 14. Healing of lifted skin of an apple flower: (*a*) epidermis, (*b*) hypodermis, (*c*) split, (*d*) callus (magnification, approx. × 700.)

The Physiological Activity of 2:6-Substituted Phenoxyacetic Acids

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SUMMARY

Anumber of 2 : 6-substituted phenoxyacetic acids in which the side chain had been substituted by alkyl groups to form α-propionic, α-*n*-butyric and α-*n*-valeric acids were investigated to assess their ability to act as growth-regulators. Besides employing standard techniques of bioassay, further experiments were undertaken to determine the effects of these compounds on the vegetative growth and development of intact plants of *Helianthus annuus*.

It has been established that all the compounds tested induced curvature in the Went pea curvature test and that without exception the activity of the parent phenoxyacetic acid (2 : 6-dichloro-, 2 : 4 : 6-trichloro-, 2 : 6-dimethyl-, and 2 : 4-dichloro-6-methyl-) was increased by side chain substitution.

In the *Avena* straight growth assay, the 2 : 6-dichloro- and 2 : 6-dimethylphenoxypropionic and butyric acids brought about statistically significant increases in length of the coleoptile sections, when measurements were made at the end of 24 hours. Their activity was of the same order as that exhibited by 2 : 4-dichlorophenoxyacetic acid.

Investigations were carried out on the uptake of water by sections of pea internode and the extension growth of *Avena* coleoptile sections when both the concentration and the length of treatment were varied. For some compounds it was found that over a certain range of concentration water uptake and extension growth were accelerated in the initial 6–8 hours. Subsequent to this, inhibition occurred so that no significant increases above control values were apparent at the end of 24 hours, and in some cases an actual loss of water or shrinkage in length took place. It was thus possible to demonstrate that 2 : 6-dimethylphenoxyacetic acid acts as a growth regulator in pea extension growth and that 2 : 6-dichlorophenoxyacetic acid is active in the *Avena* test.

The changes in the leaf area of individual pairs of leaves, together with the lengths of the internodes and shoot weight were followed after application of measured amounts of each compound to the first pair of leaves of *H. annuus*. A number of the 2 : 6-compounds were capable of modifying growth in ways similar to 2 : 4-dichlorophenoxyacetic acid. However, much higher concentration had to be applied in order to produce effects comparable to those caused by the 2 : 4-dichloro- compound. Measurements of the percentage of applied compound which penetrated into the leaf showed that there were no marked differences in this respect between the different phenoxy acids. On the other hand, experiments in which the treated leaves were left on the plant for varying periods of time led to the conclusion that the 2 : 6-substituted compounds are less readily translocated than 2 : 4-dichlorophenoxyacetic acid.

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INTRODUCTION

DURING the last two decades it has been established that many synthetic compounds have the power at low concentrations to regulate plant growth in ways similar to those of natural auxins. Concomitant with these discoveries many attempts have been made to correlate such physiological characteristics with chemical structure. While the original concepts of Koepfli, Thimann, and Went (1938) have undergone some modifications, it is still clear that for auxin-like activity a ring structure containing a double bond, a certain special relationship between the ring and the side chain, and an aci-grouping on the side chain may be considered as basic requirements.

Muir, Hansch, and Gallup (1949, 1950) in their studies of phenoxyacetic acids, have produced evidence that at least one of the ring positions ortho to to the side chain must be unsubstituted if the compound is to be active. This work has been extended and confirmed by other investigators and so far no compound of this type has been found to possess the true properties of growth regulation. Fawcett, Osborne, Wain, and Walker (1953) have shown that the substitution of a methyl or ethyl group for an alpha hydrogen atom in the side chain of certain phenoxyacetic acids, i.e. 2-chlorophenoxyacetic acid or phenoxyacetic acid itself, may considerably increase the biological activity of the compound, but no data are available which relate to the activity of 2 : 6-substituted phenoxyacetic acids similarly substituted in the side chain.

A number of workers (e.g. Audus and Shipton, 1952; Bennet-Clark and Kefford, 1954; Blackman, Osborne, and Novoa, 1954) have demonstrated clearly that in the standard tests employed for the estimation of biological activity no critical assessment should be based on either a single test or a single reading taken at the end of some selected experimental period—usually 24 hours. In either the *Avena* or the pea extension growth tests, there are conditions where sections of tissue may first increase in length or volume and then contract again during the course of the experiment. In consequence, the final value for growth stimulation may give misleading information resulting in active compounds being classed inadvertently as inactive.

With these considerations in mind, a number of 2:6 ring-substituted phenoxyacetic acids have been prepared, some with methyl, ethyl or butyl substitutions in the side chain, and a systematic investigation has been carried out to determine if any of these compounds show activity in the accepted methods for estimating growth-regulating activity.

EXPERIMENTAL METHODS

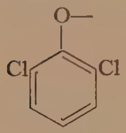
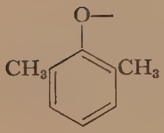
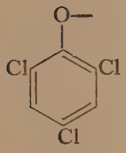
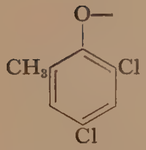
Compounds and methods of preparation

The phenoxy acids used in the investigation, together with some of their physical properties, are listed in Table I. The method of preparation involved condensing the sodium salt of the appropriate highly purified phenol with an α-bromoalkylcarboxylic ester followed by hydrolysis to the acid (Synerholm and Zimmerman, 1945). Compounds 2, 4, 8, 9, and 10 were obtained from

Pal Chemicals Limited, who used a similar preparative method, and each subsequently underwent an exhaustive purification before use. Phenol contamination in all compounds was reduced to a minimum by subjecting the acids to steam distillation at a high pH, followed by extraction from ether solution with sodium bicarbonate. In every case repeated crystallization at all convenient points in the preparation ensured that impurities were reduced to a minimum.

TABLE I

Some physical properties of the aryloxycarboxylic acids investigated

Ring structure	Compound number and name	m.p. °C. (Uncorrected)	Solvent	Crystal form
	1. 2 : 6-dichlorophenoxy-acetic acid	133–4	benzene	needles
	2. α-(2 : 6-dichlorophenoxy)-propionic acid	105–6	60–80° petrol	,,
	3. α-(2 : 6-dichlorophenoxy)-*n*-butyric acid	67–68	30–35° ,,	plates
	4. 2 : 6-dimethylphenoxy-acetic acid	134–6	benzene	,,
	5. α-(2 : 6-dimethylphenoxy)-propionic acid	56	30–35° petrol	needles
	6. α-(2 : 6-dimethylphenoxy)-*n*-butyric acid	70–71	60–80°	prisms
	7. α-(2 : 6-dimethylphenoxy)-*n*-valeric acid	73–74	,,	,,
	8. 2 : 4 : 6-trichlorophenoxy-acetic acid	177–9	benzene	needles
	9. α-(2 : 4 : 6-trichlorophenoxy)-propionic acid	113–14	60–80° petrol	,,
	10. 2 : 4-dichloro-6-methyl-phenoxy-acetic acid	180–3	benzene	needles
	11. α-(2 : 4-dichloro-6-methyl-phenoxy)-propionic acid	111–13	60–80° petrol	,,

Methods of biological assay

Went pea curvature test (*Went, 1934*). Seed of *Pisum sativum* (var. Alaska) was planted in deep enamel dishes containing sterile sand and the dishes placed in an air-conditioned dark room maintained at a constant temperature of 24·5° C. After emergence the plants were illuminated daily for half an hour with red light of 560–630 mμ. When the third internode was approximately 4–5 cm. long, sections 3·5 cm. were taken from just below the crook slit centrally and in the plane of the crook to a distance of 3 cm. from the apex and floated in a petri dish in 40 ml. of a solution of the growth substance made up in 0·005M phosphate buffer at pH 5·1. Seven sections were used per treatment, and each treatment was done in triplicate.

In these experiments the final angle of inward curvature obtained at the end of 24 hours was measured in the manner described by Went and Thimann (1937).

Pea extension growth test. The procedure for raising and sampling the plants was identical to that for the curvature test. One section—1 cm. long—was cut from each plant just below the crook. Batches of 10 sections were weighed on a torsion balance and then floated on 40 ml. of a solution of the growth substance made up in 0·005M phosphate buffer at pH 5·1. At intervals of time sections were removed from the solutions, rapidly blotted and weighed on the torsion balance, and then returned to the solution. It has been shown (Blackman, Osborne, and Novoa, 1954) that under these conditions increase in water content of the sections bears a direct relationship to the increase in length of the sections.

Avena extension growth test. De-husked seed of *Avena sativa* var. Victory (Svålof) was soaked for 24 hours at 24·5° C. and sown in shallow trays of sterile sand at 24·5° C. and the emerging seedlings given daily two half-hour periods of red illumination (560–630 mμ). When the coleoptiles were some 3 cm. high, one 5 mm. section was cut from each coleoptile from 3 mm. below the tip. The first leaf was expelled, the sections threaded on to fine glass rods and floated on the surface of 40 ml. of solution containing the growth substance made up in 0·005M phosphate buffer at pH 5·1. Ten sections were used per treatment with two or three replicates, and each experiment repeated at least twice.

Either at the end of 24 hours or, in the case of time-course experiments, at intervals of 2 hours up to 24 hours, the increase in length of the sections following treatment was measured under a microscope fitted with a micrometer.

Changes in vegetative growth of Helianthus annuus *induced by droplet applications.* The techniques employed were similar to those described by Blackman and Robertson-Cuninghame (1954, 1955) in their previous studies of the effects of indolylacetic acid and 2 : 4-dichlorophenoxyacetic acid on the growth of *H. annuus.* Briefly, plants were raised in pots to a stage where the third pair of true leaves was just visible. Then drops of an aqueous solution of the potassium or triethanolamine salts were applied with a micro-pipette to the lamina of each of the first pair of leaves. By varying the concentration of the solution or the number and size (0·0025–0·01 ml.) of drops the quantities applied ranged from 6·25 to 1,600 μg. per plant. Observations of epinasty and growth modifications were made at intervals up to the time when the plants were finally harvested at the end of 9–10 days. The final measurements included leaf area of the individual pairs of leaves, the length of the internodes, and the dry weights of the different parts of the plant.

Apart from the assessment of the growth changes, experiments were carried out to determine whether there were major differences in the rate at which compounds penetrated into the leaf. The procedure was to place 10 drops of 0·005 ml. of a known solution of the triethanolamine salt on one leaf of the first pair and after either a few minutes or 6 hours the surface residues were washed off with water. On each occasion the successive washings were bulked, made up to a standard volume, and the quantity of compound estimated by means of a Hilger spectrophotometer using a wavelength of 2,880 Å. To avoid diurnal effects the droplets were always applied in the morning. In the several experiments, for each compound there were nine sets of observations.

EXPERIMENTAL RESULTS

The Degree of Pea Curvature induced by Different Compounds

For the individual compounds the relationships between concentration and the degree of induced curvature are shown in Figs. 1, 2, and 3. For comparison, comparable data for 2 : 4-dichlorophenoxyacetic acid have been included. From inspection it is apparent that all the compounds tested are active. Thimann (1952) has previously reported that 2 : 6-dichlor and 2 : 4 : 6-trichlorophenoxyacetic acids are active in pea curvature assays, but it is clear from Figs. 1–3 that substitution in the side chain markedly increases this activity in all the series tested. 2 : 6-dichlorophenoxyacetic acid itself has a moderately high activity over a narrow concentration range, but the propionic acid appears to possess the maximum activity of this series. In the case of the 2 : 6-dimethylphenoxy acids, where the unsubstituted acetic compound is only weakly active, the activity is maximal when the chain length is extended to the *n*-butyric acid.

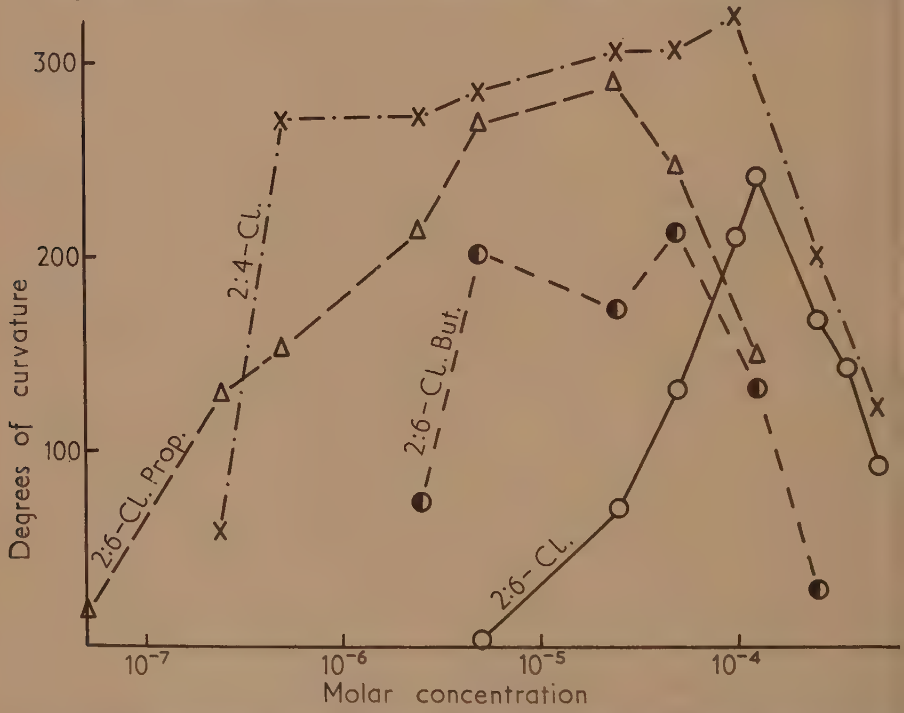


FIG. 1. Went pea curvature test. Degrees of curvature induced by different concentrations of 2 : 6-dichlorophenoxyacetic, propionic, and *n*-butyric acids compared with 2 : 4-dichlorophenoxyacetic acid.

The degree of extension growth of Avena *coleoptiles induced by different compounds*

The changes in extension growth of *Avena* coleoptiles brought about by the 2 : 6-dichlorophenoxy and 2 : 6-dimethylphenoxy series of acids are given in Fig. 4. Neither the acetic nor the propionic derivative in the 2 : 4 : 6-trichlorophenoxy or 2 : 4-dichloro-6-methylphenoxy series showed any growth-stimulating activity and have not therefore been included. It will be observed that 2 : 6-dichlorophenoxyacetic acid shows no positive activity at the end of 24 hours, whereas both the propionic and butyric compounds exhibit a highly significant activity over a wide range of concentrations. In the 2 : 6-dimethyl series, both the propionic and butyric acids show a significant but low level of activity. As in the pea test, the activity falls off again with the *n*-valeric substitution.

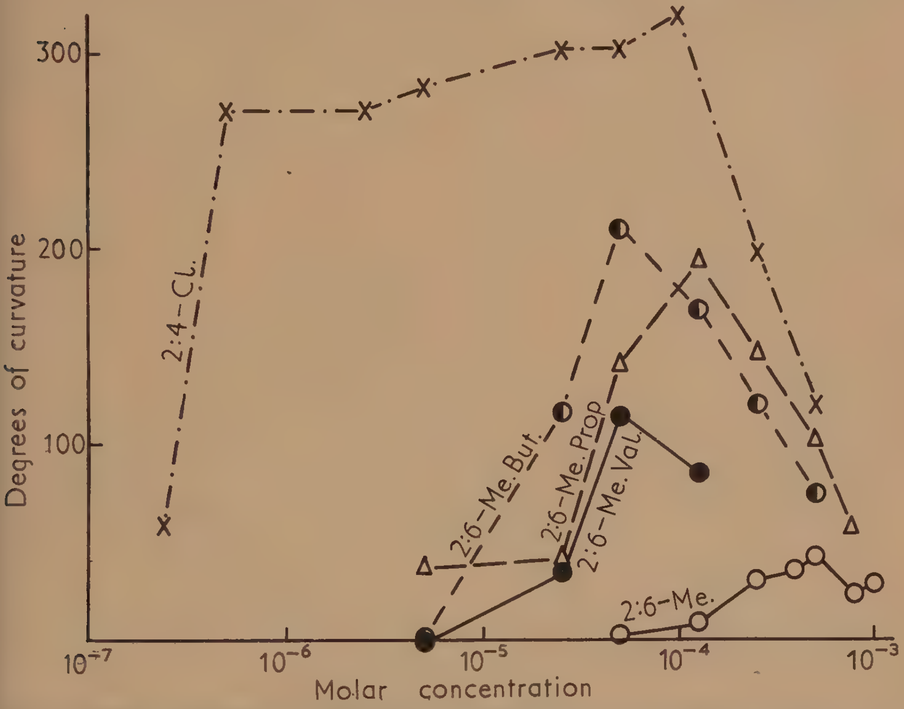


FIG. 2. Went pea curvature test. Degrees of curvature induced by different concentrations of 2 : 6-dimethylphenoxyacetic, propionic, *n*-butyric, and *n*-valeric acids compared with 2 : 4-dichlorophenoxyacetic acid.

The interactions between length of treatment and concentration on the induced changes in extension growth

The effects of varying periods of treatment at several concentrations on the activity of 2 : 6-dimethylphenoxyacetic acid were investigated by measuring the fresh weight of pea stem sections at intervals up to 24 hours. The results for selected concentrations are given in Fig. 5; it is clear that at 200 and 1,000 p.p.m. an increase in water uptake took place during the first 6 or 7 hours. At 1,000 p.p.m. some phytotoxic action then came into play, extension growth

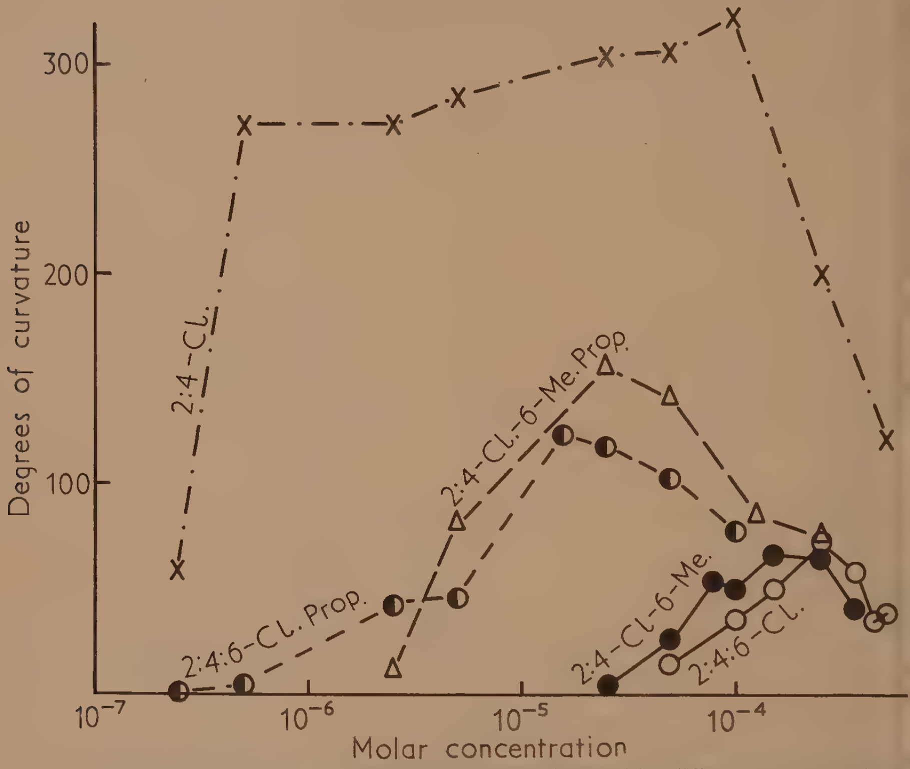


FIG. 3. Went pea curvature test. Degrees of curvature induced by different concentrations of 2 : 4 : 6-trichlorophenoxyacetic and propionic, and 2 : 4-dichloro-6-6-methylphenoxyacetic and propionic acids compared with 2 : 4-dichlorophenoxyacetic acid.

ceased, and between 12 and 24 hours relative to the control water was lost from the sections with a resultant shrinkage in length. At 200 p.p.m. the growth rate fell off after 12 hours, and the final measurements at the end of 24 hours did not differ significantly from the control.

In a similar manner 2 : 6-dichlorophenoxyacetic acid was tested in the *Avena* straight-growth test, but only measurements of the length of the sections were made. The results are shown in Fig. 6. Here again, at the higher concentrations, extension growth is accelerated for a period of 6 hours, followed by a toxic action causing the sections to shrink. 2 : 6-dimethyl, 2 : 4 : 6-trichloro, and 2 : 4-dichloro-6-methylphenoxy acetic acids were tested in a similar way, but no growth stimulation was detected over a range of concentrations from 1–100 p.p.m.

The relationship between chemical structure and the induced changes in the vegetative growth of H. annuus

In an initial and exploratory experiment the potassium salts of eleven 2 : 6-substituted phenoxy acids were compared with 2 : 4-dichlorophenoxyacetate over a common range of dosage: that is, the amounts of material applied as droplets to the first pair of leaves were 12·5, 25, 50, 100, 200, and 400 μg. per plant. At the end of 10 days the lengths of the individual internodes were measured, and the changes in fresh and dry weight of the shoot estimated.

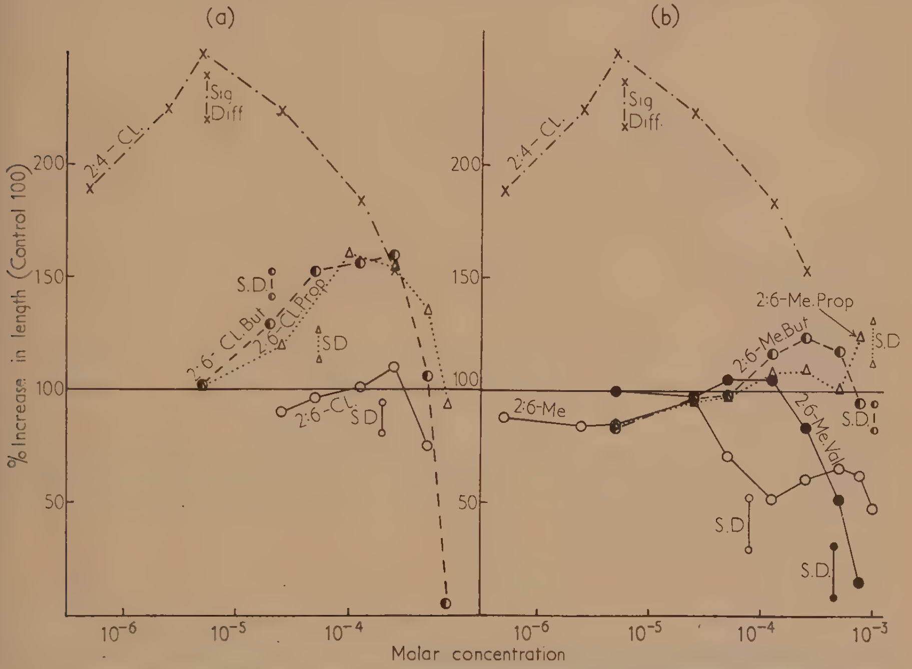


FIG. 4. *Avena* extension growth test. Extension growth induced by various concentrations of (*a*) 2 : 6-dichlorophenoxyacetic, propionic, and *n*-butyric acids, and (*b*) 2 : 6-dimethylphenoxy-acetic, propionic, *n*-butyric, and *n*-valeric acids. For comparison 2 : 4-dichlorophenoxy-acetic acid is included in both figures.

Inspection of the extensive data showed that for several of the compounds there were no significant effects, while for others the effects were only appreciable at the two highest dosages. In contrast, even 12·5 μg. per plant of the 2 : 4-dichlorophenoxyacetate caused marked reductions in the length of the internodes and some depression in shoot weight. In subsequent experiments, therefore, it was decided to investigate only the most active compounds at much higher doses. Since it was observed that some of the potassium salts crystallized out on the leaf surface quickly and thereby probably differentially affected the rate of penetration, the more soluble triethanolamine salts were substituted and a hygroscopic surface active agent added to all solutions—0·3 per cent. of 'Tween 20'.

In the first of the main experiments the range of dose for the 2 : 6-substituted phenoxy acids was 100, 200, 400, 800, and 1,600 μg. per plant, while for 2 : 4-dichlorophenoxyacetic acid the amounts were 6·25, 12·5, 25, 50, and 100 μg. per plant. The effects of these treatments on internode length are given in Fig. 7. For the first internode increasing doses of all the 2 : 6-substituted compounds had progressively arrested the extension in length, but it is to be noted that even at 1,600 μg. per plant the degree of inhibition is in no instance greater than that caused by 6·25 μg. of 2 : 4-dichlorophenoxyacetic acid.

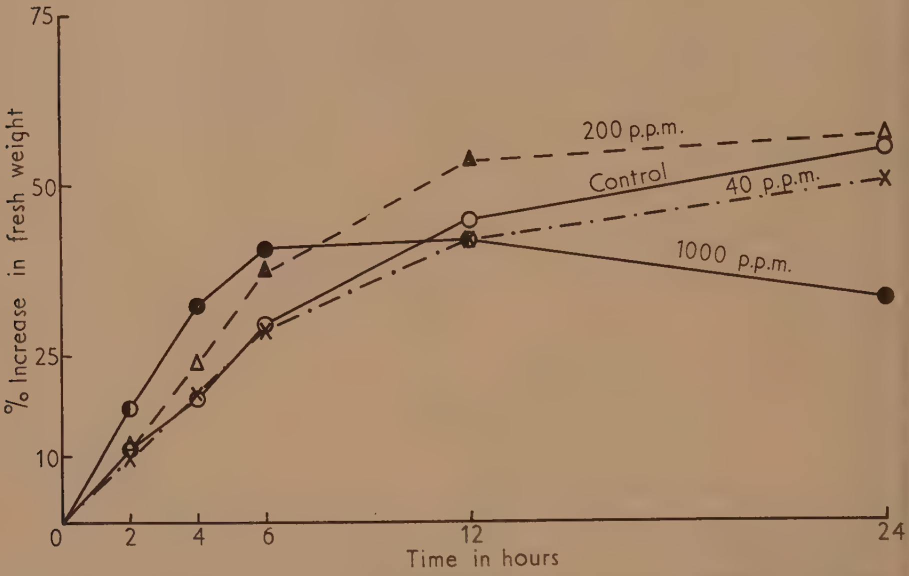


FIG. 5. Pea extension growth test. Changes in fresh weight induced by different concentrations of 2 : 6-dimethylphenoxyacetic acid at different time intervals. In the statistical analysis readings transformed into arc sign values. The blacked in points on the graph indicate values which differ significantly (P = 0·05) from the control.

In the second internode, however, the reponses induced by the 2 : 6-substituted compounds clearly differ from those induced by 2 : 4-dichlorophenoxyacetic acid. Doses of 100 and 200 μg. of 2 : 6-dichlorophenoxypropionic acid per plant brought about an increase in internode length, while doses above this failed to produce any significant effect. All doses of the butyric acid increased internode elongation; the maximal gain in length occurred at 400 μg. per plant, while higher doses produced progressively smaller increases. Neither the 2 : 6-dichloro nor the 2 : 6-dimethylphenoxyacetic acids induced any gain in internode length over that of the control, and for doses of 400 μg. and above there was a significant depression of elongation. Once more it is pertinent to observe that it only requires 6·25 μg. per plant of triethanolamine 2 : 4-dichlorophenoxyacetate to arrest almost completely the expansion of this internode.

At the time when the experiment was concluded no expansion of the third internode had occurred in control plants, while in those treated with all doses of the 2 : 6-dichlorophenoxypropionic and the three highest doses of the butyric acid, marked extension had taken place.

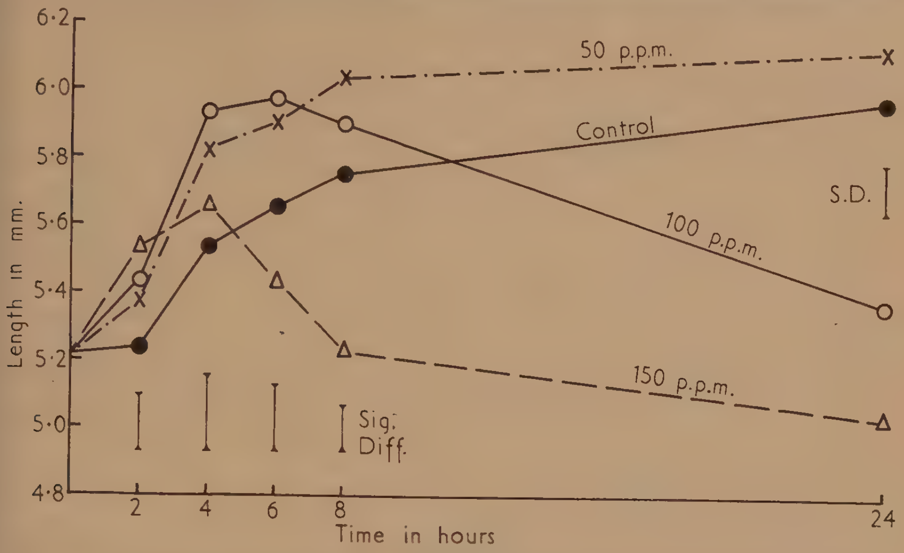


FIG. 6. Avena extension growth test. Extension growth induced by 2 : 6-dichlorophenoxyacetic acid after increasing periods of time.

Further examination of the data showed that, compared with the controls, all the compounds caused some inhibitions of expansion of the first, second, and third pair of leaves, as well as a reduction of the increase in dry weight of the shoot. By graphical methods it was possible to determine the approximate

TABLE II

The equi-effective amounts of individual compounds required to halve (a) *the leaf area of the first, second, and third pair of leaves;* (b) *the length of the first and second internode; and* (c) *the gain in shoot weight during the experimental period*

Compound (triethanolamine salt)	Equi-effective amounts—μg. plant					
	Leaf area			Internode length		Shoot dry weight
	1st	2nd	3rd	1st	2nd	
2 : 6-dichloro-						
phenoxyacetic acid	600	680	81	230	350	550
phenoxypropionic acid	740	1,250	63	270	>1,600	1,070
phenoxybutyric acid	>1,600	1,520	73	1,250	>1,600	>1,600
2 : 6-dimethyl-						
phenoxyacetic acid	910	1,590	94	320	400	1,300
2 : 4-dichloro-						
phenoxyacetic acid	50	11	3	4	3	19

amounts which would be required to halve the leaf area of each pair of leaves or to cause a 50 per cent. reduction in the increase in shoot weight during the experimental period of 10 days. These equi-effective amounts are given in Table II, together with the corresponding amounts required to halve the length of the first and second internodes.

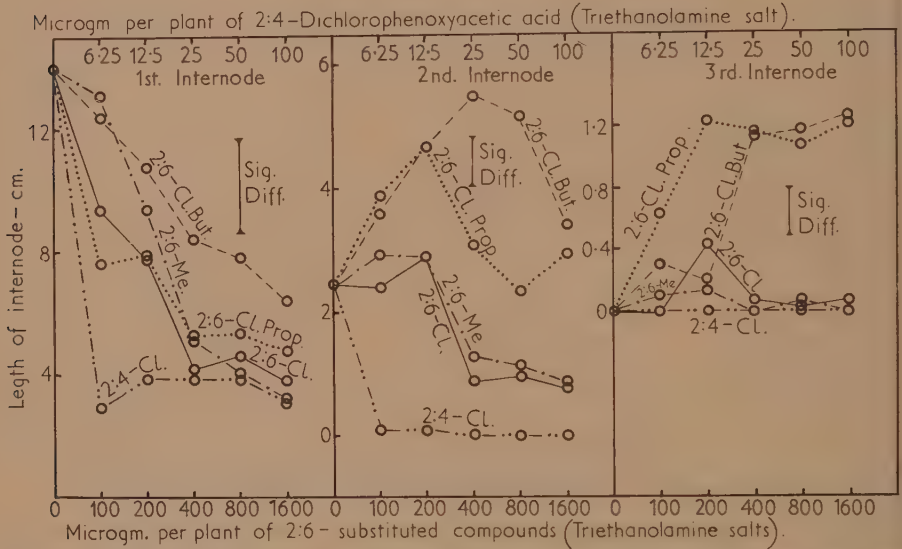


FIG. 7. Changes in the length of the first, second, and third internodes resulting from applying different amounts of 2 : 6-dichlorophenoxyacetic, propionic, and *n*-butyric acids, 2 : 6-dimethylphenoxyacetic acid and 2 : 4-dichlorophenoxyacetic acid to the first pair of leaves.

Taking firstly all the criteria together, the disparity between the phytotoxicity of 2 : 4-dichlorophenoxyacetic acid and the remaining compounds is outstanding. Next in order comes 2 : 6-dichlorophenoxyacetic acid, while the least inhibitory is 2 : 6-dichlorophenoxybutyric acid. When the data are examined on the basis of the individual criteria, it is found that the relative effectiveness of the compounds to depress growth differs considerably for different parts of the plant. Thus 2 : 4-dichlorophenoxyacetic acid is 12 to more than 32 times more effective than any of the 2 : 6-compounds in depressing by half the leaf area of the first (or treated) pair of leaves; it is 62 to 145 times more effective in halving the area of the second pair of leaves, and only 21 to 33 times more effective in the third pair. For the internodes the factor varies from 58 to 313 for the first internode, from 117 to more than 530 for the second internode, while for the shoot dry weight the factor is from 29 to more than 84.

When these results were obtained it was considered that they were consistent with the hypothesis that 2 : 6-substituted phenoxy acids were less freely transported out of the leaf than 2 : 4-dichlorophenoxyacetic acid and that their subsequent accumulation in the different tissues was also linked with a varying degree of effective translocation in the shoot. To investigate this point a series of experiments was conducted in which the treated leaves were removed at varying time-intervals after the droplet applications.

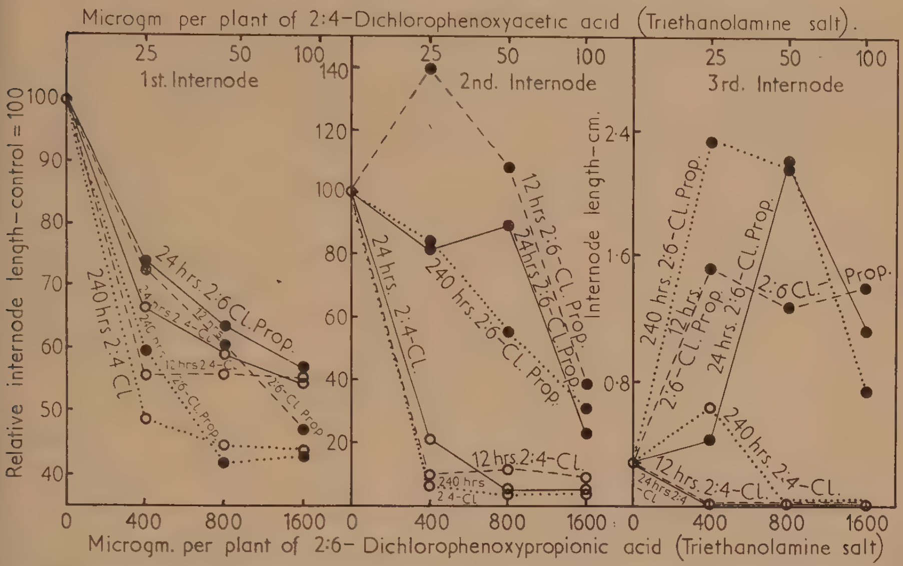


FIG. 8. Changes in the length of the first, second, and third internodes resulting from applying different amounts of 2 : 6-dichlorophenoxypropionic acid and 2 : 4-dichlorophenoxyacetic acid. The treated leaves were either left intact or removed 12 or 24 hours after the application.

In Fig. 8 are given the results of one such experiment in which the first pair of leaves were either left on the plant or cut off 12 or 24 hours after the application of 2 : 4-dichlorophenoxyacetic acid or 2 : 6-dichlorophenoxypropionic acid. For the first internode all treatments have markedly reduced the length, while irrespective of leaf removal the degree of inhibition induced by 25–100 μg. per plant of 2 : 4-dichlorophenoxyacetic acid is of the same order as that brought about by 400 to 1,600 μg. of the phenoxypropionic acid. The results for the second and third internode are in sharp contrast. While for the 2 : 4-dichloro compound the expansion in length has been almost completely inhibited under all combinations of dose and time of leaf removal, for the phenoxypropionic acid there is a marked interaction between the amount applied and the time before severance. Thus, for the second internode a combination of 12 hours and 400 μg. per plant has induced a gain in length, while higher doses or longer times are in general inhibitory. All combinations of amount and time of leaf removal have induced a lengthening of the third internode. The increase is, however, least when the leaf is cut off after 12 hours, and for the intact plant the greatest stimulation is at the lowest dose.

These results suggest that irrespective of the time the leaves are left on the plant, the amount of 2 : 4-dichlorophenoxyacetic acid transported out of the leaves and its subsequent distribution in the shoot are such that highly inhibitory levels of 2 : 4-dichlorophenoxyacetic acid can accumulate in the first, second, and third internodes. On the other hand, the data for 2 : 6-dichlorophenoxypropionic acid on a comparative basis indicate that translocation is slower.

A further experiment along the same lines was subsequently carried out in which 2 : 6-dichlorophenoxypropionic acid and 2 : 6-dimethylphenoxyacetic acid were applied at 400, 800, and 1,600 μg. per plant and the 2 : 4-dichloro-compound at 25, 50, and 100 μg. per plant. The first pair of leaves were either left on the plant for the duration of the experiment (9 days) or removed at 6, 12, or 24 hours after the application of the droplets. In general, the induced changes in the growth rate of the shoot and the length of the internodes were small and only in the case of the first internode were the effects of 2 : 6-substituted compounds appreciable. The relationship between the time of leaf removal and the mean percentage reduction in the length of this internode caused by the three compounds over all concentrations are seen in Table III. It is to be noted that the effect of extending the interval before leaf removal is more marked with the 2 : 6-substituted compounds.

TABLE III

The effects of varying the interval between the droplet application and the removal of the treated leaves on the percentage reduction in length of the first internode

Compound (triethanolamine salt)	Percentage reduction in length period before removal—hours 6	12	24	216
2 : 6-dichlorophenoxypropionate	17	24	25	30
2 : 6-dimethylphenoxyacetate	11	17	12	23
2 : 4-dichlorophenoxyacetate	32	32	30	39

The rate of penetration into leaves from droplet applications

It was thought that in the experiments with *H. annuus* the great disparity between the physiological activity of 2 : 4-dichlorophenoxyacetic acid and the 2 : 6-substituted compounds might in part be due to differences in the amounts penetrating into the leaves after the droplet applications. Accordingly, three experiments of the same type were undertaken and the percentages of the applied material which could not, after 6 hours, be removed from the leaf by washing are shown in Table IV. It is evident that when the level of absorption is high (Experiments 1 and 2) there are no major differences between compounds in percentage absorption. However, in the third experiment the conditions of late autumn would seem to have militated against rapid entry and here the 2 : 4-compound has apparently penetrated less readily. Too much should not, however, be read into these results since other work has shown that the rate of penetration changes when signs of cell injury beneath the droplet are visible to the eye. In consequence, differences of penetration between compounds may depend on the conditions governing the onset of necrosis.

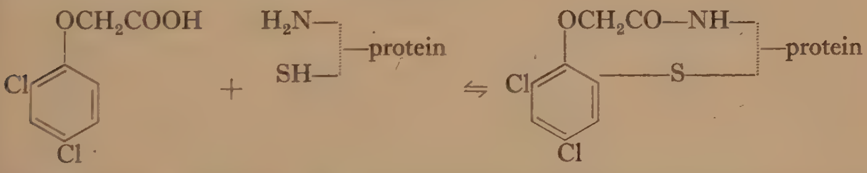
TABLE IV

The percentage penetration into the leaf 6 hours after droplet applications of the compounds to the leaf surface

	Expt. 1 24.vii.53		Expt. 2 1.viii.53		Expt. 3 15.x.53	
Compound (triethanolamine salt)	Amount applied µg.	% absorbed	Amount applied µg.	% absorbed	Amount applied µg.	% absorbed
2 : 4-dichloro-phenoxyacetic acid	62·5	25	62·5	27	1,000	2
2 : 6-dichloro-phenoxyacetic acid	..	..	..	..	1,000	3
phenoxypropionic acid	1,000	39	1,000	29	..	..
phenoxybutyric acid	..	..	..	..	1,000	4
2 : 6-dimethyl-phenoxyacetic acid	1,000	28	1,000	21	1,000	7

DISCUSSION

Recent concepts of the mode of action of growth-regulating substances have been founded on the assumption that a chemical combination takes place between the growth regulator and a specific substrate within the cell. Workers have differed in their views of the number of points of attachment. Hansch, Muir, and Metzenberg (1951) have suggested that reaction with the substrate may take place at two points; at the carboxyl group and at the 2- or 6-ring position. They postulate that the reaction may be with a cystein-like part of a protein, in the following manner:



Although the concept of 'di-ortho blocking' has fitted the results previously obtained for phenoxyacetic acids and substituted indoleacetic acids it has not been found to hold for benzoic acids, substituted in the 2 and 6 positions. A number of these compounds have been shown to possess high activity when both ortho positions are substituted with halogen groups (Muir and Hansch, 1951). From a consideration of the resonance of these molecules these investigators have suggested that electro-negative groups are readily displaced from benzoic acids, leaving a free and reactive ortho position which may combine with the substrate to give a ring-sulphur bonding with the liberation of chloride ions. The accumulation of chloride within the tissue and in the external solution has been shown to occur when *Avena* sections are placed in solutions of active 2 : 6-dichlor-substituted benzoic acids, but whether a similar increase could take place when sections are placed in solutions of a weakly active compound such as 3 : 4-dichlorobenzoic acid has not been investigated.

Nor has it been demonstrated that the release of chloride ions is proportional to the growth stimulation induced by the compound. Muir and Hansch further deduce that electropositive methyl groups in the ortho position would not readily be displaced in this manner and, in general this has been borne out by experimental work, for the benzoic acids substituted with 2 or 6 methyl groups are, at the most, only weakly active.

A consideration of the present results confirms the previous conclusion (Osborne, Blackman, Powell, Sudzuki, and Novoa, 1954) that at least some 2:6-substituted phenoxyacetic acids must be regarded as true growth-regulators. It has been demonstrated (Figs. 1, 2, and 3) that all the eleven compounds are active in the pea curvature test and even in the more stringent *Avena* coleoptile method appreciable increases in extension growth have been obtained with 2:6-dichlorophenoxypropionic and 2:6 dichlorophenoxybutyric acids and to a lesser extent with the corresponding 2:6-dimethyl compounds (Fig. 4). When the interrelationships between concentration and the duration of treatment are further investigated, it has been found that 2:6-dimethylphenoxyacetic acid can stimulate the uptake of water and extension growth of pea tissue (Fig. 5), while 2:6-dichlorophenoxyacetic acid can bring about an increase in length of *Avena* coleoptiles (Fig. 6) These results are at variance with the conclusions of Foster, McRae, and Bonner (1952) and McRae and Bonner (1952) that diortho-substituted phenoxyacetic acids only function as anti-auxins. If 2:6-substituted compounds were restricted to this role, then under conditions where the level of endogenous auxin is limiting extension growth, as in pea internodes or *Avena* coleoptiles, the induced effects should always be inhibitory at all concentrations. Indeed, a specific reaction at the 2- or 6-ring position would no longer appear to be a pre-requisite to growth-regulation, especially since compounds with electropositive or electronegative groups in these positions may exhibit activity.

So far, no record is available of a compound not possessing a carboxyl group, or a grouping readily convertible to carboxyl, which is active in tests for *Avena* and pea straight growth. It is suggested, therefore, that a partially unsaturated ring system and a carboxyl group are the basic requirements for growth-regulation, and that providing certain physical properties are obtained, which will allow the substance to penetrate the cell and reach the site of action within the cell—perhaps at a mitochondrial surface—a wide range of ring- and side-change substitutions may permit activity. In view of the wide variety of chemical substances which have so far been shown to exhibit auxin-like growth-regulating activity, it would seem that the physical properties of the molecule as a whole, as well as the specific chemical groupings, may be responsible for determining the final degree of activity shown by the substance. In this connexion two of us (Blackman and Novoa) studying some twenty variously substituted phenoxyacetic acids have obtained evidence that the ability to induce a standard degree of chlorosis in *Lemna minor* is dependent on both the physical characteristics and chemical structure.

It is relevant to observe that in the pea curvature or *Avena* coleoptile tests the concentrations producing the maximal response for some of the more active 2 : 6-substituted compounds (e.g. the 2 : 6-dichlorophenoxypropionic and butyric acids) are of the same order or show less than a twentyfold difference from the corresponding concentrations for 2 : 4-dichlorophenoxyacetic acid. On the other hand, for the equi-effective concentrations required to halve leaf area or internode length, the ratio may range from 12 to more than 32 for the first pair of leaves to over 530 for the second internode (Table II). The data of Table IV, although not conclusive because of the variations due to necrosis and because the analytical technique did not allow quantities below 60 μg. of 2 : 4-dichlorophenoxyacetic acid to be accurately investigated, nevertheless, do not suggest that variations in percentage penetration into the leaf can account for the differences between compounds in the equi-effective concentrations. In the interpretation of Figs. 7 and 8 and Table III it has been concluded that the differences between the 2 : 6-substituted compounds and 2 : 4-dichlorophenoxyacetic acid may in part be due to differences in the rate of transport out of the leaf and the subsequent movement in the shoot. This conclusion presupposes that the basic physiological modes of action are common to all compounds and that it is either the compounds themselves or similar breakdown products which are transported. Since 2 : 4-dichlorophenoxyacetic acid always inhibited the growth of the second and third internodes (Figs. 7 and 8) while the 2 : 6-dichlorophenoxypropionic and butyric acids at some concentrations induced internode extension, it could be advanced that the compounds were not all acting in the same manner. On the other hand, Blackman and Robertson-Cuninghame (1954, 1955) have demonstrated that in *H. annuus*, 2 : 4-dichlorophenoxyacetic acid can, like the 2 : 6-compounds, accelerate the growth rate of the second internode, while depressing the growth rates of the first internode and the leaves. They have emphasized that at the present stage of knowledge an adequate explanation of these complex effects cannot be provided, and that different physiological systems are seemingly involved in the different parts of the plant. The same considerations are clearly applicable to the 2 : 6-substituted phenoxyacetic acids.

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The Effects of X-rays on the Embryonal Growth and Development of *Hordeum vulgare* L.[*]

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SUMMARY

The effects of softer X-rays as an external agent on the developing embryos were manifested by a number of abnormalities such as enlargement and vacuolation of cells, disturbances in cell walls, disintegration of cells leading to the formation of cavities, disturbances in distribution of meristematic tissues, a number of proliferations in different parts of the embryo, and retarded growth of the embryo during the early post-radiation period. In addition to these, deeply stained globular cytoplasmic bodies were observed in radiated embryonic cells. Very similar kinds of abnormalities were produced by softer X-rays starting from 400 r. and including 800 r. The frequency of occurrence and degree of proliferations in embryos irradiated with 800 r., however, were relatively higher than those produced by 400 r. and 500 r. Treatments with 300 r. units of irradiation induced only the appearance of deeply stained globular bodies within cytoplasm and no effects were visible with dosages below 300 r. Comparative measurements of cell-lengths of the treated and control embryos at undifferentiated as well as at differentiated stages showed an increase in cell-lengths within radiated embryos. Radiated embryos showed defective pattern of germination. Harder X-rays were found to be less effective in inducing embryonic abnormalities than softer rays when equal calculated dosages of the two were applied to the flowering spikes of *Hordeum vulgare* L.

INTRODUCTION

THE science of plant embryology is customarily traced back to Aristotelian time (384–322 B.C.), but embryological study in the present form actually began with the publication of J. Hanstein's famous memoirs on the development of monocotyledonous and dicotyledonous embryos in 1870 (from Johansen, 1950). Hanstein described the sequence of segmentation and development of embryos, grown under natural conditions, starting with the fertilized eggs and concluding with mature stages of the embryos. Until recently embryology consisted mainly of these descriptions of embryonic development plus some embryological studies comparing different groups of plants. In 1924 a new field of embryological study, experimental embryology, came into existence. Generally in this kind of study very tiny embryos are excised from ovaries and are then grown as artificial cultures. Their further

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growth and development are followed in order to determine the nutritional requirements of the embryos during early stages of growth. Thus far only one study has dealt with the effects of external agents on the growth and development of embryos growing intact on plants (Eunus and Mericle, 1954). Investigations concerning such effects open up a new and interesting branch of embryological study. With this view in mind X-radiation was chosen as an external agent to investigate its effects on the embryonal growth and development of *Hordeum vulgare* L.

MATERIALS AND METHODS

Materials used in this investigation were obtained from plants grown from seeds of *Hordeum vulgare* var. 'Mars' furnished by the Farm Crops Department of the Michigan State College through the courtesy of Dr. K. Frey. The variety 'Mars' is an inbred line of long-day plant.

Raising of plants. Three sets of experiments were carried out, two during the spring and autumn of 1953 and the third in the winter of 1954. Plants were grown on soils (a mixture of one part vermiculite, one part sand and three parts loam) inside a temperature, light, and humidity control room. Nutrients were added to soil three times during each set of experiments from stock solutions each of which contained one of the following salts dissolved in a litre of distilled water: 14·6 g. potassium phosphate, 68·9 g. calcium nitrate, 7·4 g. ammonium sulphate, 69·9 g. magnesium sulphate, and 1·6 g. ferrous sulphate. Each plant during the course of development received on the average, 2 ml. of ferrous sulphate solution and 9 ml. of each of the remaining four nutrients.

Plants flowered about 9 weeks after germination. The time of pollination was recorded and 3 to 4 days later plants were grouped, labelled, and then transferred to the X-ray room for radiation treatment. Immediately following irradiation plants were returned to the control-room and allowed to grow to full maturity.

Radiation. A therapeutic X-ray machine (G. E. Max. 250 III) was used for the radiation. This was calibrated with a standardized Victorian r-meter. The X-ray beam was produced by the following factors: 200 kilo-volt of power, 15 milliampere of current, inherent filtration of 3 mm. aluminium, and a 0·25 mm. copper and 1 mm. aluminium added filter. Further characteristics were: 0·75 mm. copper half-value layer, 50 cm. focal spot distance, and 400 cm.^{2} beam size.

Plants of the first set were divided into 8 groups, 7 of which were given exposures of 50, 100, 150, 200, 250, 300, and 350 r. units of X-rays respectively, while the eighth was kept as a control. In each case radiation treatment was given by a single exposure.

Plants of the second set were divided into 9 groups, 8 of which were treated with 100, 200, 300, 400, 500, 600, 700, and 800 r. of X-rays respectively with a group being kept as the control. As with the previous case, single exposure was given to flowering spikes of each group, but the X-ray machine was somewhat altered for the proper accommodation of plant heads within the area o

X-ray beam. The cone (29 cm. long) which is usually a part of the machine for therapeutic treatment, was removed, thus reducing the focal spot distance. X-rays used in the second set of experiments, therefore, were softer. At the time of treatment a lead sheet 3 cm. thick was used to cover each group of plants being irradiated, so that exposure of neighbouring groups to X-rays could be avoided.

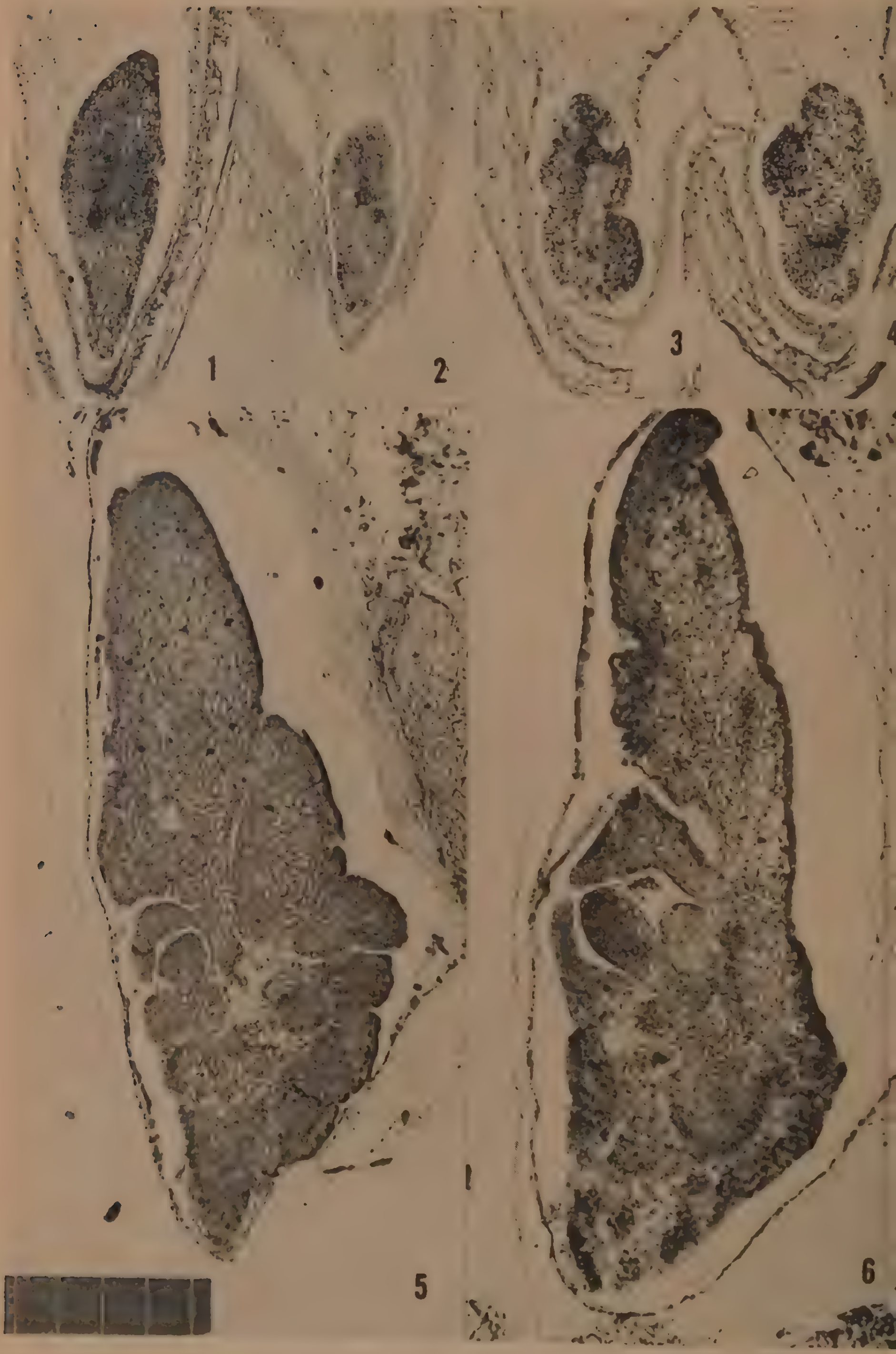
Since the second set of experiments showed some interesting effects, it was decided to group plants of the third set in various stages of embryonic development and treat them with 500 r. units of irradiation during their development. Accordingly, 4 groups of plants were selected for treatment on 4 dates, approximately 2, 5, 8, and 10 days following fertilization time. Further, to aid in determination of the threshold dose, two sets of four groups each were selected to be radiated with 150, 200, 250, and 350 r. on two dates about 5 and 8 days after fertilization. Plants were exposed to radiation in the same manner as in the first set of experiments using harder X-rays. An additional group of plants were treated with 800 r. and another group was kept as control.

Sampling and slide preparation. In the first and second sets of experiments a few ovaries in each group were killed and fixed immediately after irradiation in order to know the probable embryonal stage of development at the time of treatment. In the first set one additional fixation was made at harvest-time, while in the second irradiated set ovaries were collected and fixed on three dates: 4, 8, and 15 days after treatment. In the case of the third set three to four ovaries from each plant belonging to each group were killed and fixed just prior to radiation treatment to record the exact stage of development at the time of exposure. Further killing and fixation of ovaries from each plant were made 6 and 12 days after treatment.

The fixative used was F.A.A. formaline, 1, acetic acid, 1, 95 per cent. ethyl alcohol, 6, distilled water, 6. At the time of fixation, glumes were removed from the ovaries. Older ovaries were treated with 1 : 1 solution of hydrofluoric acid and 50 per cent. ethyl alcohol for 5 to 7 days to soften the fruit-seed-coat and to facilitate infiltration. Standard methods of dehydration with ethyl-butyl alcohol mixtures and paraffin embedding were followed. Paraffin blocks were cut at thicknesses of 7 to 16 microns, depending on the materials used and their stages of development.

Most of the slides were stained with Chlorazol Black E and counterstained with Fast Green. Some of the slides were stained with Safranin-Crystal Violet-Fast Green. Chlorazol Black E was found to be more suitable for photomicrography. A few slides were hydrolysed with N-hydrochloric acid for 5 minutes in a 58° C. oven and then stained by the Feulgen technique in order to verify whether or not any Feulgen positive bodies developed within the cytoplasm of treated embryonic cells.

Measurements. For comparative study of cell-lengths of treated (500 r., softer X-rays) and control embryos two sets of measurements were taken from undifferentiated (young) and differentiated (mature) embryos. The first set involved random measurements of 500 cells taken from 15 slides each of treated



FIGS. 1–6. Effects of softer X-rays (500 r.); one scale division = 0·01 mm.; 1 and 2, 4 days; 3 a
4, 8 days; 5 and 6, 15 days after irradiation

and control undifferentiated embryos, while in the second set 250 cells from each part (cotyledon, coleoptile, first leaf, and coleorhiza) of the differentiated ones were similarly taken.

Germination. Control seeds and those irradiated with 400, 500, 600, 700, and 800 r. in the second set of experiments were germinated in paper towels in order to note any variation in germinability caused by irradiation. Five days after the complete germination of control seeds, final countings of all germinated and non-germinated seeds were made.

OBSERVATIONS

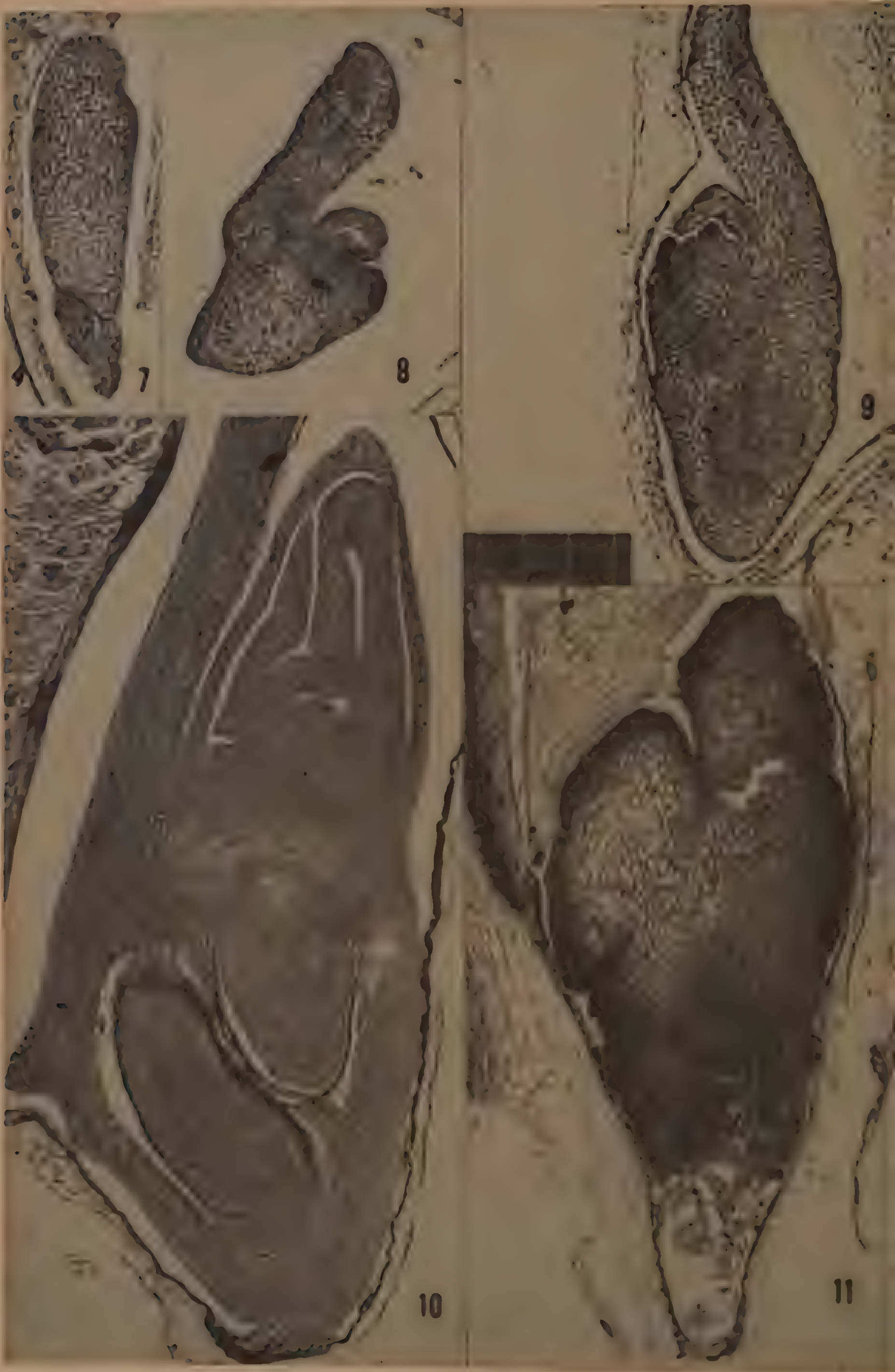
Harder X-rays were used in the first and third sets of experiments, while softer rays were used in the second set. Since the first set of experiments were performed mainly for exploratory purposes and produced no visible effects, description will include only observations made in the second and third sets of experiments. Furthermore, since effects produced by softer and harder rays were not same, the two will be described separately.

Effects of softer X-rays. Single exposures of each of 100, 200, 300, 400, 500, 600, 700, and 800 r. were given to flowering spikes. Visible morphological variations were found in embryos treated with irradiation as low as 400 r., while 300 r. of X-rays produced only cytoplasmic effects and no visible changes were induced by dosages lower than 300 r. Cytoplasmic effects were manifested by the appearance of numerous deeply stained globular bodies within the cytoplasm. Similar effects were observed also in embryos treated with higher dosages. These globules proved to be Feulgen negative, so presumably do not contain DNA.

The kinds of abnormalities observed were almost identical in embryos irradiated with X-rays starting with 400 r. and including 800 r. A general description of all abnormalities obtained from irradiation, therefore, will be given under 500 r. followed by brief remarks about the effects induced by 800 r.

Effects with 500 r. In a comparative study of all slides it was apparent that the effects of X-rays were not uniform on all embryos of the same spike. Embryos exhibited a wide range of variable irradiation-effects. Some embryos were highly affected and showed profuse proliferations in later stages, some behaved almost like normal embryos, while others were intermediate types between the two extremes.

X-ray effects also varied, depending on the stage of development of the embryos at the time of irradiation. Younger embryos appeared more sensitive to X-rays than older ones. Embryos radiated after differentiation showed no abnormalities four days after treatment, while visible abnormalities were evident at the corresponding time in those which were treated before differentiation. These findings conform with the 'Bergonie-Tribondeau Law' (Bergonie and Tribondeau, 1906) which holds that the sensitivity of cells varies directly with their reproductive capacity and inversely with their degree



FIGS. 7–11. Normal and irradiated embryos. One scale division = 0·01 mm.

of differentiation, i.e. younger embryos are more sensitive to radiation than older embryos.

All cells of the same embryo generally were not equally affected (Figs. 2–4 and 14–15). In some cases cells of a treated embryo were enlarged and vacuolated more or less uniformly (X-ray effects, in this case, were presumed equally distributed). In the majority of cases, however, differential effects were found in different parts of an embryo and as a result of these effects scattered 'patches' of densely staining cells were produced. These 'scattered patches' of tissues were surrounded by vacuolated cells and ordinarily appeared to contain even denser cytoplasm than the corresponding regions in the control embryos. These were apparently regions of high metabolic activity and behaved like 'apical meristems' giving rise to a number of proliferations. Once scattered 'meristematic' tissues were established, growth occurred in various directions and produced outgrowths at different places. There was no definite place, sequence, or regularity for the development of such outgrowths. Their occurrence and direction of growth probably depended directly on the place of origin of the 'scattered meristems' and indirectly on the passage of X-rays and their absorption. That portion of the embryo where absorption of X-rays was relatively high but unequal showed many such 'scattered meristems' which in turn produced proliferations (Figs. 2–6 and 13–17). The frequency of such proliferations, however, was higher at the region near the base of the cotyledon and directly opposite the plumule or nearby. Some of the proliferations at this region resembled a second plumule of the embryo (Fig. 5).

The first visible X-ray effects were enlargement and vacuolation of cells. In some cases enlargement and vacuolation led to a disintegration of cells, and lysigenous cavities were formed. The formation of cavities in this way appeared to occur at any place in an embryo either in cotyledon, coleoptile, hypocotyl, or in the root regions. Disintegration of cells which led to the formation of cavities possibly occurred as a result of greater X-ray absorption in the specific region, while absorption of lesser amounts presumably led only to cell enlargement and vacuolation. Occurrence of such cavities was noted to be relatively more frequent in regions of the hypocotyl and root (Figs. 5 and 7) and less frequent in regions of root-cap, coleorhiza, and suspensor. It should not be stated, however, that regions of coleorhiza and suspensor were less susceptible to X-rays. Figures 7 and 11 show that the basal region of the embryo may also be severely affected.

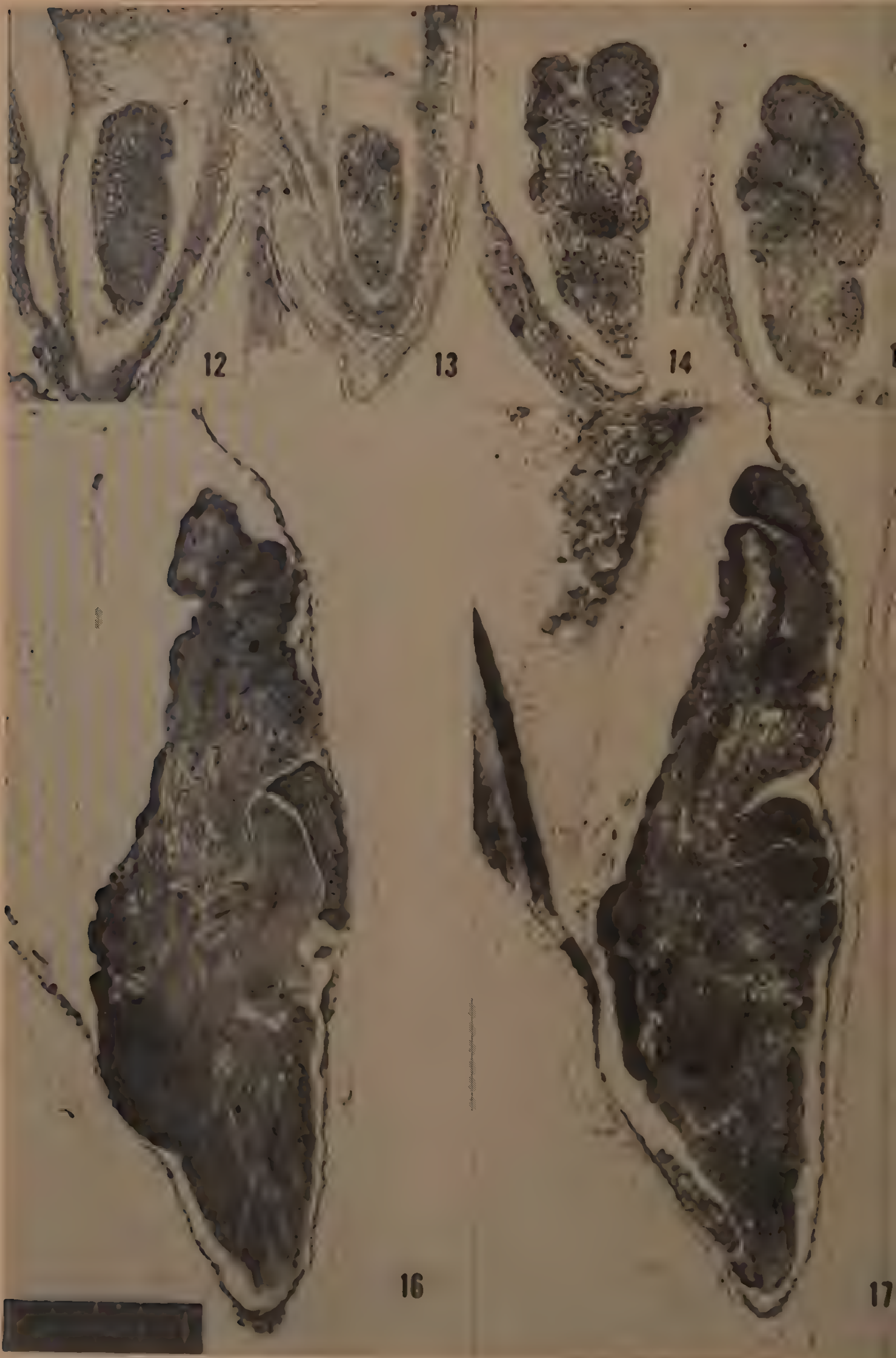
FIG. 7. Embryo X-rayed with 500 r. of softer rays and collected 8 days after treatment. The basal end of the embryo is severely affected.

FIG. 8. Embryo radiated with 800 r. of harder X-rays and collected 6 days after treatment. Cells are enlarged and vacuolated.

FIG. 9. Differentiated control embryo. Approximately the same age as those of Figs. 3–4 and 14–15.

FIG. 10. L. S. of mature control embryo with seminal roots developed. It is the same age as those of Figs. 5–6 and 16–17.

FIG. 11. Embryo treated with 500 r. of softer X-rays and collected 15 days after radiation. The basal part is highly affected and other parts show proliferations.



FIGS. 12–17. Effects of softer X-rays (800 r.). One scale division = 0·01 mm.

While individual cell-sizes increased, general growth as well as development of radiated embryos appeared to be retarded. The treated embryos were smaller and less differentiated than the control of the same age. The controls were not only larger but also had differentiated into cotyledon, coleoptile, plumule, root, root-cap, and coleorhiza (Figs. 3, 4, and 9). Treated embryos, however, seemed to regain the rate of growth and appeared almost of the same size as the control when collected fifteen days after treatment, although at that time they lacked complete differentiation (Figs. 5, 6, 11, 16, and 17).

The embryos which were older at the time of treatment or which were affected more or less uniformly by X-rays, showed growth and development somewhat different from what has been described in the foregoing paragraphs. Four to eight days after irradiation embryos were observed to be composed of somewhat enlarged and vacuolated cells but with an almost normal arrangement of meristematic regions in them (Figs. 1 and 2). There were practically no proliferations in these embryos. The time of embryonal differentiation was similar to that of the control. Individual cells were mostly filled with denser cytoplasm with numerous deeply stained globular bodies. Some cells were seen with two nuclei. Occasionally a few small cavities or gaps were found scattered within the embryo.

Not only were X-ray effects dependent on the degree of absorption of X-rays and the age of embryos as a whole, but they also appeared to vary with the age of the individual parts. Thus the treatment of X-rays prior to differentiation of the embryo seemed to produce more effects on the coleoptile than on the first leaf, while radiation at the time of differentiation of the embryo produced almost similar effects on the two. The primary root usually showed somewhat irregular and retarded growth, while seminal roots grew like those in the control embryos. This differential effect probably was due to the fact that irradiation is likely to produce more effects in embryo-parts which are at their primordial stage of development or at least close to that stage.

Retarding effects of irradiation on growth was almost uniform on all parts of the embryo. The first leaf of the plumule, however, appeared to be relatively thicker, and showed greater growth than the other surrounding parts and at times seemed to project through the plumule sheath (Figs. 6, 16, and 17).

Effects Induced by 800 r. It has been mentioned that similar kinds of abnormalities were produced by X-rays of dosages from 400 r. to 800 r. The frequency of occurrence and degree of proliferations in embryos radiated with 800 r., however, were relatively higher than those produced by 400 r. and 500 r. (Figs. 13–17). Furthermore, 800 r. seemed to produce more physico-

FIG. 12. A uniformly affected embryo collected 4 days after irradiation. Cells are enlarged and vacuolated.

FIG. 13. Unequally affected embryo (observed 4 days following treatment) showing enlargement and vacuolation of cells and origin of 'scattered patches' of denser cytoplasmic tissues.

FIGS. 14–15. Embryos collected 8 days after irradiation. Vacuolation, enlargement, and degeneration of cells and proliferations are visible.

FIGS. 16–17. L. S. of older embryos (15 days after treatment) showing proliferations, lysigenous cavities, and also enlarged vacuolated cells.

chemical after-effects in cells which resulted in 'unhealthy' cells of unusual appearance even in differentiated embryos. It also became apparent that the latent period required in producing visible variations in radiated embryos appeared less in the case of 800 r. dosage.

Comparison of cell-lengths of control and treated embryos (800 r.). Relative cell-lengths were measured in order to obtain an indication of comparative growth of treated and control embryonic cells. Measurements were taken separately prior to gross differentiation of the embryo as well as at the mature stage of development. In the latter case cell-lengths of different parts were measured in order to obtain a closer comparison. Data obtained show that following irradiation there had been a considerable increase in cell-lengths. In the case of undifferentiated embryos the average length of treated cells was $13{\cdot}31 \pm 0{\cdot}14$ units, while that of the control was only $6{\cdot}52 \pm 0{\cdot}08$ units. As embryos continued to grow, the relative difference in cell-lengths between control and radiated embryonic cells diminished, although it remained statistically highly significant. Average cell-lengths measured $11{\cdot}8 \pm 0{\cdot}16$ in the coleorhiza, $10{\cdot}86 \pm 0{\cdot}09$ in the cotyledon, $8{\cdot}18 \pm 0{\cdot}11$ in the coleoptile, and $6{\cdot}5 \pm 0{\cdot}09$ in the first leaf of treated differentiated embryos, while average cell-lengths were $6{\cdot}98 \pm 0{\cdot}07$, $8{\cdot}64 \pm 0{\cdot}15$, $5{\cdot}70 \pm 0{\cdot}11$, and $4{\cdot}94 \pm 0{\cdot}7$ units in the corresponding regions of the control. Thus while mean lengths of treated cells in cotyledon, coleoptile, and first leaf were 1·3 times larger than control cells in the corresponding parts, those in the coleorhiza were about twice the size of the control. Less reduction in cell-lengths in the coleorhiza during further growth and development can probably be interpreted by assuming that relatively fewer cell divisions occurred in this region.

Germination tests. For want of sufficient number of seeds only 14 seeds belonging to each group were used in germination tests. Although no dependable conclusions can be drawn from these tests, they may, however, give some indication about the effects of X-rays on the pattern of germination. Data obtained from the tests show that while all 14 control seeds germinated, only 2 seeds treated with 400 r. and one in each of the 500 r. and 600 r. groups showed normal germination. In addition to this, some treated seeds showed partial growths. Some seminal roots during germination emerged from 6 more seeds irradiated with 400 r., 4 of which exhibited growth of plumule while one showed growth of the main root (radicle). Almost similar conditions were met with seeds treated with 500 r. and 600 r. In the case of seeds treated with 700 r. and 800 r. neither radicle nor plumule emerged while seminal roots appeared from four of the fourteen seeds during germination. It may thus be inferred that X-rays were relatively more effective in inhibiting the growth of the main root (radicle) during germination, while seminal roots were less affected in this respect.

Effects of harder X-rays. All the four exposures of 500 r. produced neither visible morphological nor cytoplasmic variability. Similarly, preparations for the purpose of determining threshold dosage did not show any irradiation effect. A dosage of 800 r. of harder X-rays, however, induced some effects.

Vacuolation and enlargement of a few scattered cells were observed when irradiated embryos were examined 6 days after treatment (Fig. 8), but even this dose did not disturb the distribution of meristematic tissues within the embryos and induced no abnormal outgrowths such as those that were characteristic of plants irradiated with softer X-rays. Treated embryos collected 12 days after irradiation showed neither enlarged vacuolated cells nor any disorganized cells. This was probably due to the fact that these vacuolated cells recovered from the shock of X-ray treatment, became densely cytoplasmic, and the empty space or spaces created by degenerated cell or cells was filled by the growth of the adjoining cells.

DISCUSSION

General retardation of growth was manifested by proportionate smallness of affected embryos. Similar effects were observed in animal embryos by a number of investigators. In plants growth retarding effects from irradiation were observed by Rasch (1951) and Quantler, Schertiger, and Stewart (1952), among others with the application of higher doses of X-rays, while a great many earlier workers (from Johnson, 1936) found stimulative influences at low dosages.

Smith and Kersten (1941) reported that during earlier post-radiation periods the radiated roots of *Vicia faba* showed growth rates similar to those of the controls, but in later stages of development their growth was retarded. In contrast to their findings this investigation shows that the growth of X-rayed barley embryos was retarded for a short time following irradiation but assumed a normal rate of growth during later stages of development.

Wilson and Karr (1951) and Wilson, Jordan, and Brent (1953) thought that the growth-retarding effects of X-rays on rats might be attributed either to reduction in mitotic rate in radiated embryonic cells or to an outright destruction of a certain percentage of cells. They further felt that both might be involved in producing the effect. A similar explanation may be plausible for the retarded barley embryos. A reduction in cell division as well as a destruction of some cells in radiated embryos were apparent during early part of the post-radiation period.

Wolcott (1937), during his study of the influence of X-rays on stem and leaf anatomy in barley, observed that cell-size increased following irradiation. He also found that cell walls appeared to lack 'reinforcement'. A similar increase in cell-size as a result of irradiation has been observed in both animals and plants by a number of other investigators. Smith and Kersten (1941, 1942) noted that an enlargement of cells was accompanied by vacuolation and that in some cases these cells disintegrate giving rise to lysigenous cavities, and in other cases schizogenous cavities arose as a result of the disorganization of the middle lamellae of the cell walls. The results of the present experiments corroborate these findings. Following irradiation, cells became vacuolated and increased in size. Affected cells often disintegrated giving rise to cavities.

Sometimes they appeared disconnected from one another apparently due to a disorganization of the middle lamellae of the cell walls.

Uniform effects of X-rays were not observed in barley embryos of the same spike. Some were highly affected, showing profuse proliferations, some behaved almost normally, while others were intermediate types between the two extremes. It was conceived that a uniform X-ray exposure to all flowers of a spike was not possible. It, however, was not expected that flowers borne on the same side of a spike would show differential radiation effects. Nevertheless, this differential effect did occur and may be due to the fact that all flowers of a spike often are not in 'exactly' the same stage of embryonic development. Similar non-uniform effects of irradiation were reported by Horlacher and Killough (1931) and Caldecott, Frolik, and Morris (1952). Horlacher and Killough (1931) while working with cotton seedlings raised from X-rayed seeds noticed that seedlings could be grouped into three classes: normal, intermediate, and dwarf. Likewise, Caldecott, Frolik, and Morris (1952) found that seedlings from X-rayed barley seeds had a wide range in distribution (height) whereas those from thermal neutron radiation had a very narrow range.

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Polyphenol Oxidase in the Respiration and the Salt Uptake of Storage Tissues

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SUMMARY

The respiration and potassium uptake of beet and potato disks have been measured in the presence of protocatechuic acid. It is found that protocatechuic acid in a concentration of 10^{-3} to 3×10^{-3} M at pH 6, stimulates both oxygen uptake and carbon dioxide output. This stimulation of respiration is accompanied by a reduction in potassium uptake.

Experiments with carbon monoxide indicate that polyphenol oxidase mediates the 'extra' respiration in the presence of protocatechuic acid, although this enzyme does not appear to play any part in the normal respiration, at least in 5 per cent. oxygen. These results are considered in relation to previous claims concerning the role of polyphenol oxidase in salt uptake and respiration of potato disks.

Potassium uptake by potato disks is light-reversibly inhibited by carbon monoxide, indicating the participation of cytochrome oxidase in salt uptake in this, as in other tissues.

INTRODUCTION

In studies of salt uptake by plant tissues much attention has been paid to the identity of the terminal oxidase mediating salt uptake and respiration. This role has been attributed to each of the three main plant oxidases in various tissues, evidence in support being drawn mainly from experiments with inhibitors. Of the inhibitors used, carbon monoxide is probably the most useful. Carbon monoxide inhibits both cytochrome oxidase and polyphenol oxidase, but the inhibition of the former is sensitive to light (Warburg, 1949). Ascorbic oxidase is not inhibited by carbon monoxide.

Cytochrome oxidase occurs in a wide variety of plants (Webster, 1952) and its role in mediating salt uptake and respiration is well established in carrot disks (Weeks and Robertson, 1950) and wheat roots (Sutter, 1950).

Neither polyphenol oxidase nor cytochrome oxidase can be extracted from 10–11-day-old barley roots and the respiration of the intact roots is not inhibited by carbon monoxide (James, 1953*a*). The respiration is inhibited by diethyldithiocarbamate, which in the absence of polyphenol oxidase is good evidence of ascorbic oxidase. Since diethyldithiocarbamate causes similar inhibition of respiration and salt uptake, mediation of salt uptake and respiration in this tissue has been attributed to ascorbic oxidase (Russell, 1954).

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Polyphenol oxidase is said to function as a terminal oxidase by catalysing the oxidation of *o*-diphenols by atmospheric oxygen. The quinones formed in this reaction are reduced by hydrogen donators in the tissue, thus maintaining the supply of *o*-diphenol for oxidation and resulting in a cyclic oxidation and reduction. The conflicting views concerning the part played by polyphenol oxidase in respiration have been fully reviewed recently (Dawson and Tarpley, 1951; James, 1953*b*); here reference will be made only to work with disks of potato tuber.

Evidence to support this function of polyphenol oxidase is derived mainly from the fact that the addition of *o*-diphenols to potato disks leads to a marked stimulation of respiration. Both oxygen uptake and carbon dioxide output increase, the R.Q. remaining close to unity (Boswell and Whiting, 1938; Baker and Nelson, 1943). Although the use of catechol has been criticized, since it causes an inhibition of respiration after a short-lived stimulation (Schade, Bergmann, and Byer, 1948), such a criticism cannot apply to the use of other diphenols, e.g. protocatechuic acid, which cause a prolonged stimulation of respiration. This, together with evidence from experiments with inhibitors, has led to the claim that polyphenol oxidase is responsible for the major part of the respiratory oxidation of potato disks (Baker and Nelson, 1943). This claim is not, however, borne out by studies using carbon monoxide as an inhibitor. Levy and Schade (1948) found that carbon monoxide light reversibly inhibits the respiration of potato disks, thus demonstrating the participation of cytochrome oxidase. This light reversal is complete, rendering the participation of polyphenol oxidase unlikely. If this is so, then if polyphenol oxidase acts as suggested above, the 'extra' respiration in the presence of *o*-diphenols is linked with an oxidase not normally concerned in respiration. This is contrary to the usual inference drawn from the stimulating action of diphenols. Conversely, if the normal oxidase system operates in this 'extra' respiration, then *o*-diphenols must act in some manner other than that supposed. Steward, Stout, and Preston (1940) have suggested that polyphenol oxidase is involved in salt uptake by potato disks on the grounds of the general parallelism of salt uptake, respiration, and oxidase activity which was observed in this tissue. In view of the evidence outlined above, casting doubt on the part played by this enzyme in respiration, it seemed of interest to investigate further the connexion of salt uptake and polyphenol oxidase, and to examine terminal oxidation in the presence of *o*-diphenols. Of particular interest was the possibility that increased respiration in the presence of *o*-diphenols might be accompanied by increased salt uptake, if polyphenol oxidase mediated these two processes.

In this study, respiration and potassium uptake of potato and beet disks have been studied in the presence and absence of protocatechuic acid. Beet disks, commonly used in this laboratory in physiological studies, have been used in this investigation since, although they contain polyphenol oxidase, they behave very similarly with regard to salt uptake to carrot disks (Robertson, Turner, and Wilkins, 1947), in which cytochrome oxidase has been definitely shown to mediate salt uptake (Weeks and Robertson, 1950). In addition, carbon monoxide has been used to study the pattern of terminal oxidation in the presence of protocatechuic acid.

EXPERIMENTAL METHODS

Preparation of the tissues. Disks were cut with a hand microtome 7·5 mm. in diameter and 0·75 mm. thick, rinsed thoroughly and aerated in distilled water for 2 to 7 days before use.

Potassium uptake. Conical flasks of about 50 ml. capacity, containing 2 or 4 ml. of the experimental solution and 20 disks (0·7–0·8 g.) were mounted on a carrier shaken in a horizontal plane at 100 strokes per minute, essentially in the manner of a Warburg apparatus. The mouths of the flasks were closed by rubber stoppers, pierced by two glass tubes projecting above the surface of the water in which the flasks were immersed.

At the end of the experimental period the flasks and disks were thoroughly rinsed with distilled water and the washings made up to 50 ml. for analysis. Alternatively, 1 ml. was removed and made up to volume: it was replaced by 1 ml. of the original solution and the experiment continued.

Potassium estimations were made by means of an EEL flame photometer or by the use of potassium labelled with ^{42}K. Radioactivity was determined by liquid counting, using an M6 counter attached to a decade scaler.

Respiration. Standard Warburg technique was used. The flasks were of conventional double sidearm type, of about 25 ml. capacity, in which were placed 2 or 4 ml. of solution and 20 disks in order that they should be comparable with the flasks used for potassium uptake. Protocatechuic acid was either tipped from the sidearm, after respiration had been measured for a preliminary period in its absence, or added directly to the disks before the experiment started. The disks were weighed before the start of the experiment and small differences in weight allowed for in calculating rates of respiration.

Carbon dioxide was measured by the direct method of Warburg, omitting hydroxide from one of a pair of vessels. 3N hydrochloric acid was tipped in from the sidearm at the end of the experiment to release carbon dioxide bound in the solution; the carbon dioxide released was added to the total before the R.Q. was calculated.

Gas mixtures. Carbon monoxide was prepared by the action of concentrated sulphuric acid on formic acid. The gas released was passed through alkaline pyrogallol, washed in acid, and collected in a 5-litre flask by displacement of water. Oxygen and nitrogen were obtained in cylinders from commercial sources. Gas mixtures were obtained by reducing the pressure of carbon monoxide or nitrogen and replacing with oxygen to a concentration of 5 per cent. A Haldane apparatus was used to check the oxygen concentration. The Warburg flasks were filled by four successive reductions of pressure to 12 cm. of mercury, refilling each time with the gas mixture. Inhibition was observed in the dark by covering the vessels with metal foil, which could be removed to test light reversal. Light reversal was brought about by means of a 250-watt mercury vapour lamp, placed as close as possible to the transparent bottom of the Warburg bath and directly underneath the flasks, which were themselves within a centimetre of the bottom.

Protocatechuic acid. Solutions were made up freshly each day because protocatechuic acid oxidizes slowly in air. The amount of N/10 alkali necessary to bring the acid solution to pH 5 was determined by titration, using a glass electrode, and the required amount added in each experiment. Two samples were used, one B.D.H. and the other L. Light & Co., without further purification.

All experiments were carried out at 25° C.

RESULTS

The effect of protocatechuic acid on potassium uptake and respiration

The first experiments were carried out in unbuffered solution but it was found that the pH drifted from 5 to 7 over the course of the experiment and this seemed to be coupled with a change in the effect of protocatechuic acid on respiration. In subsequent experiments, buffer solutions were used and the pH measured at the end of each experiment.

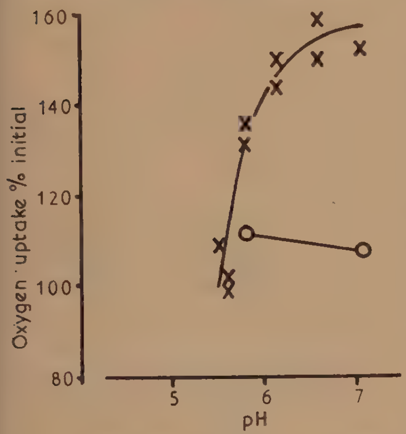

FIG. 1. The effect of pH of M/15 phosphate buffer on the response of beet disks to protocatechuic acid 3×10⁻³ M. Oxygen uptake plotted as a percentage of the initial rate in buffer. (×——×) in protocatechuic acid, (○——○) in buffer alone.

Respiration experiments were first carried out using M/15 phosphate buffer. In the experiment shown in Fig. 1, beet disks were placed in phosphate buffer, and after one hour, further buffer or protocatechuic acid (3×10^{-3} M) was added from the side arm. Oxygen uptake from 1–1½ hours after addition (expressed as percentage of the rate in the hour before addition) is plotted against the final pH of the solution. On the addition of buffer alone, an increase in oxygen uptake of about 10 per cent. occurred, but when the protocatechuic acid was added the reaction varied greatly with pH. At pH 5·6 the rate was the same as that in the initial period, but at pH 7 an increase of more than 50 per cent. occurred. In another experiment a range of concentrations of protocatechuic acid was applied to beet disks at 2 pH. In Fig. 2 oxygen uptake in protocatechuic acid is plotted as a percentage of the rate of the disks in buffer alone. At the lower pH, a slight stimulation, perhaps not significant, occurred at a concentration of 3×10^{-3} M but there is considerable inhibition at the higher concentrations. At pH 7, there was stimulation in all concentrations above 10^{-3} M.

Since the pH of the solution influences so greatly the effect of protocatechuic acid on the respiration of these disks, it is clearly important to consider pH when potassium uptake is measured. In potassium uptake experiments it is not possible to use strong buffers because the increase in ion concentration renders the amount absorbed by the disks very small in relation to the amount left in solution, and hence difficult to measure with any great degree of accuracy. An attempt was made to control the pH drift by adding to the 0·01 M potassium chloride normally used potassium dihydrogen phosphate 0·01 M in concentration and adjusted to the required pH with potassium hydroxide. It was found that these solutions drifted from an initial pH of 5 to pH 6 over the course of a few hours, but no change occurred from an initial pH of 7.

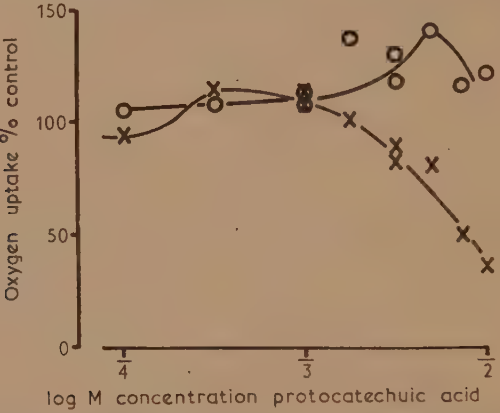

FIG. 2. The effect of a range of concentrations of protocatechuic acid on the respiration of beet disks at two pH. Oxygen uptake plotted as percentage of controls without protocatechuic acid. (X——X) pH 5. (O——O) pH 7.

In Table I the results of a series of experiments using dilute buffered solutions are given; in every case but one potassium uptake is reduced in protocatechuic acid.

TABLE I

Potassium uptake by beet disks in the presence (+) or absence (—) of 3×10^{-3} M protocatechuic acid

	Potassium uptake μ equivs./gm. f.wt.			
	pH 5–6		pH 7	
Experiment	—	+	—	+
1	32·2	20·7	37·0	16·4
2	29·3	21·1	31·3	20·6
3	29·0	13·1	26·1	15·9
4	41·0	30·5	29·0	28·2
5	41·2	26·6	37·9	29·4

To compare potassium uptake and respiration in buffer solutions of the same concentration, further experiments were carried out. Potassium uptake was measured as in the previous experiment and respiration measured on replicate sets of disks in the same solutions in the Warburg apparatus. The solutions were placed directly on to the disks (instead of tipping from the side arm of the Warburg flask) and respiration measurements started after equilibration. Where beet disks were used (Fig. 3), potassium uptake was inhibited by both 10^{-3} M and 3×10^{-3} M protocatechuic acid at pH 6 but respiration was stimulated. Using potato disks (Fig. 4), a very marked inhibition of potassium uptake (in fact actual slight loss of potassium in 3×10^{-3} M protocatechuic acid) was found. Nevertheless respiration is stimulated.

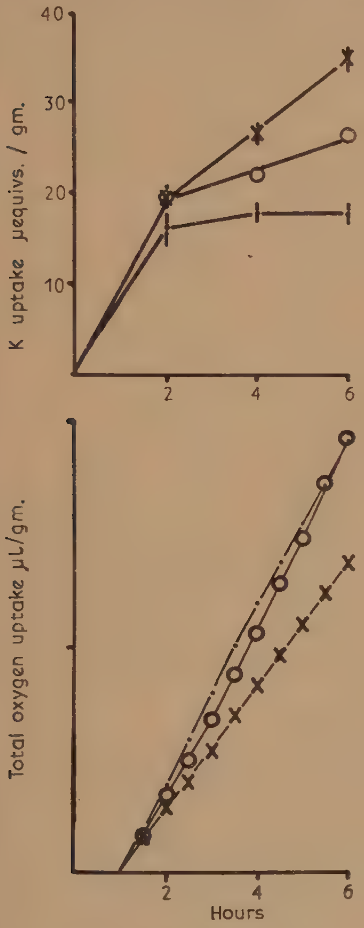

FIG. 3. Potassium uptake and respiration of beet disks in protocatechuic acid. (X——X) control, (O——O) 10^{-3} M, (•——•) 3×10^{-3} M. pH 6·0. Vertical lines indicate standard errors.

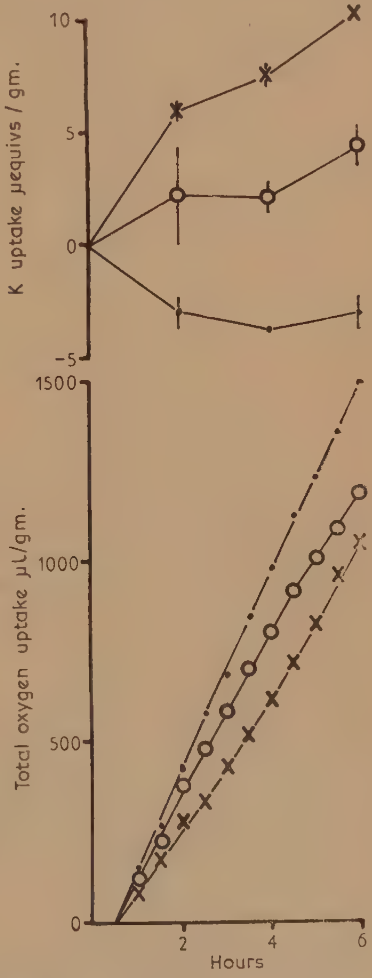

FIG. 4. As in Fig. 3, but with potato disks.

In the experiments reported in Figs. 4 and 5, both oxygen uptake and carbon dioxide were measured. In Table II total gas exchange over the whole of the experimental period, except the initial equilibration period, is shown. In spite of a marked stimulation of oxygen uptake the R.Q. falls only slightly.

TABLE II

Total gas exchange (μl./g. fr. wt) of disks in dilute buffer with or without protocatechuic acid, pH 6

protocatechuic acid concn.	O_2	CO_2	R.Q.
(*a*) *Beet, 5 hours*			
0	−678	772	1·14
	−602	678	1·13
10^{-3} M	−861	952	1·11
	−957	1,071	1·12
3×10^{-3} M	−968	1,030	1·06
	−936	973	1·04
(*b*) *Potato, 5½ hours*			
0	−1,032	1,178	1·14
	−1,060	1,193	1·13
10^{-3} M	−1,185	1,360	1·15
	−1,205	1,359	1·13
3×10^{-3} M	−1,492	1,538	1·03
	−1,431	1,558	1·09

The increased respiration which follows the addition of protocatechuic acid to beet or potato disks is not accompanied by increased potassium uptake, and these experiments do not support the suggestion that polyphenol oxidase is connected with salt uptake in these tissues. The possible causes of the inhibition of potassium uptake which was found will be discussed below.

The terminal oxidase mediating the 'extra' respiration

The carbon monoxide studies of Levy and Schade (1948) suggest that polyphenol oxidase is not concerned in the respiration of potato disks in the absence of added *o*-diphenols. The experiments described below were carried out to discover whether polyphenol oxidase was concerned in any part of the respiration in the presence of protocatechuic acid. The experiments were carried out with potato disks only, since the red pigment of beet disks interferes with light reversal of inhibition.

The following treatments were used: Air, Nitrogen 95 per cent./Oxygen 5 per cent. (referred to as N), Carbon monoxide 95 per cent./Oxygen 5 per cent. in the dark (CO-dark) and in the light (CO-light). Preliminary experiments showed that the oxygen uptake of potato disks was inhibited by CO-dark but that the inhibition disappeared on transference to CO-light. Inhibition could be reimposed by redarkening the flasks. In subsequent experiments separate flasks were used for light and dark treatments instead of transferring flasks from dark to light or vice versa. It was found that disks in CO-light respired at a mean rate of 91 (81–109) per cent. of disks in N, compared with 45 (30–56) per cent. for disks in CO-dark. Increase in the light intensity by a factor of 4 did not result in increased light reversal. The high degree of inhibition by CO-dark is characteristic of one-day-old potato disks, which were used because Schade and Levy (1949) found that potato disks became less sensitive to CO as they aged in water. Unfortunately these disks, showing a high degree of sensitivity to CO, do not respond readily to the addition of protocatechuic acid. Stimulation of respiration by protocatechuic acid (3×10^{-3} M at pH 6·0) was variable, the rate of oxygen uptake in N in protocatechuic acid ranging from 100–140 per cent. of that of disks in N without protocatechuic acid. In CO-light the rate of oxygen uptake was about the same in 5 out of 6 experiments in the presence or absence of protocatechuic acid. This suggests that the 'extra' respiration is sensitive to CO in the light, but the stimulation by protocatechuic acid in N was not sufficiently great to render the results unequivocal.

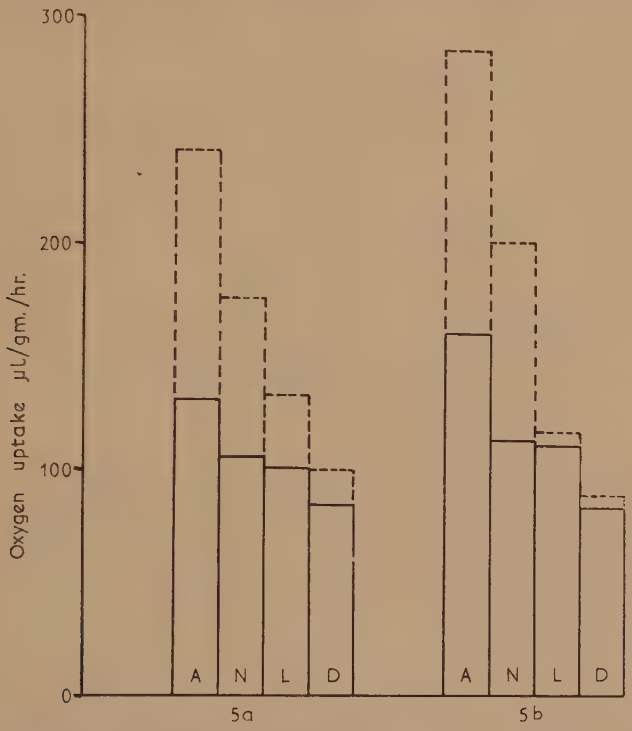

FIG. 5. Respiration of potato disks in various gas mixtures in the presence (– – – – –) and absence (———) of protocatechuic acid, 3×10^{-3} M in M/15 phosphate buffer pH 6·0. 5*a*, protocatechuic acid added before the gas mixtures; 5*b*, vice versa. A, air; N, nitrogen/5% oxygen; L, CO-light, D, CO-dark.

It was found that a greater stimulation could be obtained by shaking 2–3-day-old disks in protocatechuic acid for some hours before treating with the gas mixtures. In one experiment (Fig. 5*a*) disks were shaken overnight in M/15 phosphate buffer pH 6·0, with and without 3×10^{-3} M protocatechuic acid. The disks were then rinsed and replaced in fresh solution of the same composition as in their preliminary treatment and respiration measured for 1 hour. The gas treatments were applied and respiration measured for a further hour. It is clear from Fig. 5*a* that protocatechuic acid causes an increase in oxygen uptake in all gas mixtures. However, although in the absence of protocatechuic acid there is little difference between N and CO-light (in fact no difference if allowance be made for slight differences in initial rates in air) a great difference exists between these two treatments in the presence of protocatechuic acid. This indicates that a considerable part of the stimulated respiration is inhibited by CO-light.

The results of a second experiment are shown in Fig. 5*b*. After preliminary shaking similar to that of the previous experiment, all disks were suspended in buffer irrespective of previous treatment and the gas mixtures applied. After one hour protocatechuic acid was added to those which had been shaken in it in the preliminary period, and buffer solution was added to those previously shaken in the absence of protocatechuic acid. In this experiment the presence of protocatechuic acid has little effect on the rate of oxygen uptake in CO-dark or CO-light while causing great stimulation in N. This sensitivity to CO-light suggests that polyphenol oxidase is acting terminally in the 'extra' respiration in the presence of protocatechuic acid.

The effect of carbon monoxide on potassium uptake by potato disks

The results reported above show that the 'extra' respirarion in the presence of protocatechuic acid is mediated by polyphenol oxidase. The fact that this increased respiration is accompanied by a decreased rate of potassium uptake does not lend any support to the suggestion that polyphenol oxidase is involved in salt uptake. The possibility that cytochrome oxidase might mediate salt uptake has been studied using carbon monoxide as a selective inhibitor.

In preliminary experiments it was found that the low rate of potassium uptake of the potato disks used made small differences difficult to measure with sufficient accuracy. To minimize this difficulty, the volume of solution was reduced to 2 ml. and potassium uptake was measured by means of labelled potassium in 0·01 M potassium chloride solution.

Gas mixtures of the same composition as those described previously were used. Potassium uptake by disks in solution in contact with air was measured at 25° and at 5° in order to estimate the absorption occurring relatively independently of metabolism. An absorption period of 6 hours was allowed with renewal of the gas mixtures after 3 hours. The results of one experiment are shown in Table III.

Potassium absorption is significantly reduced by lowering the oxygen concentration from that of air to 5 per cent. There is no significant difference between N and CO-light but both cause significantly less reduction of uptake than CO-dark. The absorption of potassium in CO-dark is not significantly different from that in air at 5°, suggesting that it is mainly due to non-metabolic absorption. The sensitivity to light of the inhibition caused by

TABLE III

The effect of carbon monoxide on potassium uptake by potato disks from 0·01 M KCl labelled with ^{42}K

Gas mixture	Temperature °C.	Potassium absorption mμ equivs./gm. f.wt.
Air . . .	25	4,014±117
N . . .	25	3,205±186
CO-light . .	25	3,034±224
CO-dark . . .	25	1,924±172
Air . . .	5	2,159±197

CO-dark demonstrates that cytochrome oxidase mediates salt uptake and respiration in this tissue also. It is interesting to note that growth of potato disks under the influence of auxins is also light-reversibly inhibited by carbon monoxide (Hackett, Schneidermann, and Thimann, 1953).

DISCUSSION

The respiration of potato disks in gas mixtures containing 5 per cent. oxygen is partially inhibited by carbon monoxide in the dark. The inhibition is reversed by light showing that cytochrome oxidase and not polyphenol oxidase is the operative terminal oxidase in this fraction of the respiration. This finding is similar to that of Levy and Schade (1949) and conflicts with the earlier conclusions of Boswell and Whiting (1938) and Baker and Nelson (1943). These were based on experiments in which *o*-diphenols were added to disks of storage tissue causing a stimulation of respiration, presumably mediated by polyphenol oxidase. It has now been shown that when respiration is stimulated by the addition of protocatechuic acid, a large fraction is sensitive to carbon monoxide in the light, and is therefore attributable to polyphenol oxidase. Thus it would appear that the addition of protocatechuic acid enables an enzyme, not normally active as a terminal oxidase, to mediate a substantial portion of the respiration. It seems probable that a high external concentration of *o*-diphenols, available as substrate, enables polyphenol oxidase to compete more successfully with other terminal oxidases than is usually the case. Although the extra respiration in the presence of protocatechuic acid is mediated by polyphenol oxidase, the further conclusion that it is the major terminal oxidase in the normal respiration of potato tissue (Baker and Nelson, 1943; Boswell, 1945) cannot be upheld. It is interesting to note that on other grounds Lerner (1954) has suggested that polyphenol oxidase might mediate the extra respiration of ivy leaves in the presence of *o*-diphenols, while playing no part in the normal respiration.

When respiration of beet and potato disks was stimulated by the addition of protocatechuic acid in a concentration of 10^{-3} to 3×10^{-3} M at pH 6, potassium uptake was inhibited. This result does not support the suggestion of Steward, Stout, and Preston (1940) that polyphenol oxidase is concerned in salt uptake by potato disks. It has been further shown that in this tissue cytochrome oxidase mediates potassium uptake since potassium uptake is light reversibly inhibited by carbon monoxide.

The cause of the inhibition of salt uptake by protocatechuic acid is unknown, though several possibilities may be suggested: (i) An inhibition of salt uptake accompanying a stimulation of respiration is reminiscent of the action of dinitrophenol (Robertson, Wilkins, and Weeks, 1951). However, a close similarity does not exist. Robertson *et al.* (1951) showed that in carrot disks dinitrophenol stimulated respiration mediated by cytochrome oxidase, which is also a terminal oxidase of the normal respiration. In potato disks protocatechuic acid stimulates respiration mediated by polyphenol oxidase, which is not operative in normal respiration. The two substances would appear therefore to have different modes of action.

(ii) A simple explanation of the effect of protocatechuic acid on potassium uptake is possible if salt uptake is as closely dependent on cytochromic oxidase activity as Lundegårdh (1952) has proposed. Were this so, it might be expected that reduction of cytochrome oxidase activity in the presence of an active polyphenol oxidase would lead to reduced salt uptake. However, recent evidence that salt uptake is not solely dependent on cytochrome oxidase activity (Overstreet and Jacobson, 1952; Russell, 1954) makes this less tenable.

(iii) Phosphorylation is known to occur in the passage of electrons from reduced diphosphopyridine nucleotide (DPNH) to oxygen through the cytochrome system (Hunter, 1951). It is also known (Link, Klein, and Barron, 1952) that DPNH may be oxidized in the presence of catechol and polyphenol oxidase. If no phosphorylation occurred in this oxidation, reduced potassium uptake might result from reduced phosphorylation when polyphenol oxidase took part in terminal oxidation following the addition of protocatechuic acid. The *o*-diphenol would be acting in an analogous manner to redox dyes which by-pass or inhibit phosphorylation (Hunter, 1951). Both this and the previous suggestion presuppose that cytochrome oxidase activity is reduced when respiration mediated by polyphenol oxidase is stimulated on the addition of protocatechuic acid. It has not been possible to determine whether this is so, since light-reversal of carbon monoxide inhibition has been used to determine cytochrome oxidase activity. In carbon monoxide, polyphenol oxidase is inhibited and plays no part in respiration whether protocatechuic acid is present or not. It cannot, therefore, be assumed that cytochrome oxidase activity is the same in nitrogen and carbon monoxide gas mixtures. A method of measuring cytochrome oxidase activity in the presence of an active polyphenol oxidase is necessary to test these suggestions fully.

(iv) Boswell (1945) has claimed that an excess of H-donator to quinone is normally present in potato disks but it seems possible that when a high external concentration of *o*-diphenol is present, this situation may be reversed. Some of the quinone produced in the oxidation of the *o*-diphenol might then take part in side reactions leading to interference with metabolic processes, including salt uptake. Quinones react with amino-acids (Boswell, 1945) and are known to inactivate succinnic dehydrogenase (Potter and Dubois, 1943).

Machlis (1944) found that iodoacetate and malonate, inhibitors of dehydrogenases, inhibited salt uptake by barley roots.

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Factors controlling Flowering in the Chrysanthemum

V. DE-VERNALIZATION IN RELATION TO HIGH TEMPERATURE AND LOW LIGHT INTENSITY TREATMENTS

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WITH TWO PLATES

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SUMMARY

Experiments are described which indicate that the annual vernalization requirement of the basal shoots of the Chrysanthemum is due to annual de-vernalization of these shoots as the main axis grows up and flowers. Plants sprayed with varying concentrations of maleic hydrazide were arrested in their growth for considerable periods, but this enforced 'dormancy' did not affect their vernalization status. This makes it appear unlikely that mere suppression of growth through apical dominance of the main shoot is the cause of this de-vernalization of basal shoots. Fully or partly vernalized plants heated to 40° C. for up to 30 hours did not become de-vernalized. Heat treatment at 35° C. for as long as 30 days also failed to achieve complete de-vernalization, but here flowering was delayed by periods equivalent to the time spent at high temperature. However, at the end of the heat treatment progress towards flowering was resumed at the normal rate. Complete de-vernalization can be brought about by prolonged exposure to low intensity illumination. This treatment appears to be effective right up to the stage when the first morphological changes leading to inflorescence formation take place. These results are discussed in relation to similar experiments on the de-vernalization of rye and *Hyoscyamus niger*.

INTRODUCTION

In the first paper of this series (Schwabe, 1950) evidence was presented that the Chrysanthemum plant has a vernalization requirement. It was also shown that the cold treatment must be given each year to the young basal shoots appearing at the end of the flowering period of the main axis. The fact that there appeared to be little or no carry-over of the vernalization stimulus from the previous season raised the question whether the basal shoots arising from lateral buds below the ground on the main flowering stems, and also second-order laterals, had never become vernalized or whether the vernalization stimulus had been lost again by the time they started to grow actively, i.e. whether they had become de-vernalized in the interval between the original cold treatment and the end of flowering of the main shoot. Evidence that the need for annual cold treatment is due to annual de-vernalization would then raise the further question of its mechanism.

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De-vernalization has been shown to occur under certain conditions in both winter annuals and biennial plants (e.g. winter rye, beet, and *Hyoscyamus niger*). In these three plants de-vernalization has been brought about by high temperature treatment (Purvis and Gregory, 1952; Owen, Carsner, and Stout, 1940; Lang and Melchers, 1947). The effectiveness of such heat treatment was shown to be limited to a short period immediately after vernalization. Heat treatment applied later than this time, which in rye and *Hyoscyamus* was as short as about 4 days, remained ineffective. With rye high temperatures also became progressively less effective as the prior vernalization became more complete, and only incompletely vernalized plants were susceptible to heat de-vernalization. Anaerobic conditions for prolonged periods at 20° C., on the other hand, caused at least a partial reversal of vernalization even in the spring form of rye —which is presumably 'genetically' fully vernalized (Gregory and Purvis, 1938). Vegis (1948 and 1949), working with winter buds of *Stratiotes aloides*, found that when these buds have reached the end of their normal rest period (about the end of November) it is possible to induce them to elongate by immersion in water of a somewhat higher temperature. But this is possible only over a narrow range of temperatures; if kept above the upper limit secondary dormancy will be induced. The range of temperatures suitable for growth, however, widens with prolonged storage in the cold, and eventually secondary dormancy can no longer be induced by heat treatment. This entry into secondary dormancy closely resembles the de-vernalization effects in rye and *Hyoscyamus*.

In order to investigate the possible occurrence of de-vernalization in the Chrysanthemum a series of experiments was begun in 1950 and the results of some of these are presented below.

METHODS AND RESULTS

The general methods of raising the plant material were identical with those described in the previous papers of this series. The short-day treatment given consisted of 8 hours daylight followed by 16 hours darkness, except where otherwise stated. The vernalization treatment was given to the young cuttings in a refrigerator kept at a mean temperature of approximately 5° C. During the vernalization treatment a light intensity of about 25 ft. c. was maintained for 8 hours per day. Leaf-number counts and the times taken for the macroscopic appearance of the inflorescence bud were used as measures of effects on flowering behaviour. Internode lengths were also determined and furnished an indication of effects on vegetative growth.

Natural de-vernalization: The first experiment to be described was designed to distinguish between the two alternative explanations for the annual vernalization requirement of basal shoots: failure of the lateral meristems to become vernalized when the whole plant was subjected to cold treatment, or subsequent loss of the effect, i.e. de-vernalization.

In this experiment young vernalized cuttings were grown and subsequently cut down to a low node (about 2–3 cm above soil level) after different stages of development had been reached by their main axes. The stages were: (1) immediately after the end of vernalization, (2) after production of 15 new leaves, (3) immediately after inflorescence initiation of the main shoot, (4) when petals showed in the inflorescence bud, (5) when the inflorescences began to wither. All axillaries above ground level were removed at the time of decapitation and only true basal shoots were allowed to grow out. A few mature leaves were left

TABLE I

Effect of state of development of main axis on de-vernalization of basal shoots. Variety 'Sunbeam'. Eight replicates

Main shoot decapitated	No. of plants with budded low laterals at time of decapitation	No. of plants with budded basal shoots after decapitation	No. of plants with only vegetative shoots after decapitation	Mean leaf number of largest basal shoot to budding	Mean leaf number of largest vegetative basal shoot	Mean height of budded basal shoots (cms.)	Mean no. of days to budding from date of decapitation
(1) Immediately after end of vernalization . .	0	8	0	21·3	. .	20·1	50·0
(2) After production of 15 new leaves . .	0	4	4	19·3	39·0	14·8	41·0
(3) Immediately after budding*. . .	0	3	5	15·0	30·1	15·4	29·3
(4) After appearance of petals* . . .	5	2	6	11·5	23·3	10·0	18·5
(5) After main inflorescence started to wither	5	1	7	7	18·6	8·0	33

* Prior to decapitation the main shoots in treatments (3)—(5) budded 27·6 days after the end of vernalization with a mean increment of 16·9 leaves.

on the stumps. As will be seen from Table I a few of the precocious laterals removed had actually budded by the time treatments (4) and (5) were decapitated. The flowering data obtained from the basal shoots developed after decapitation are also shown in Table I. All eight replicates decapitated at an early stage produced flowering shoots from below soil level. The number of flowering basal shoots produced after decapitation in the other treatments declined steadily as the cutting down of the main shoot was delayed. In treatments (4) and (5) taken together only three plants produced minute basal shoots which became budded after decapitation. On the other hand, all 16 plants in these two treatments produced a crop of wholly vegetative shoots, which were clearly de-vernalized.

A similar experiment was then set up in order to observe the behaviour of low lateral apices above soil level. In this experiment the plants were again decapitated to the same low node and single axillary shoots—always from approximately the same level—were allowed to grow out. The data are shown in Table II and demonstrate that lateral meristems on the main shoot are in fact vernalized (treatment (1)) in the same way as the truly basal shoots of the previous experiment. However, it is also clear from the data in this experiment that the lateral buds allowed to grow out did not become de-vernalized.

Especially after the inflorescences of the main shoot were fully developed, flowering of these low laterals occurred with quite low leaf numbers. The failure of these low laterals to become de-vernalized suggested that there is in fact a difference between them (and also the usual few low laterals growing up and flowering almost simultaneously with the main shoot) and the true basal shoots developing on the underground stolons, since the latter need to be vernalized again, as was shown before (Schwabe, 1950). The existence of such a difference had been suspected earlier as the explanation why Čajlachjan (1937) had met with no difficulty in his experiments demonstrating basipetal translocation of the flowering stimulus. It seems probable that the accelerated flowering seen in treatments (4) and (5) Table II is due to such translocation of the flowering stimulus from the main apex, but such translocation seems to become operative only after full initiation of the main apex has occurred (cf. also Schwabe, 1954).

TABLE II

Effect of state of development of main axis on de-vernalization of lower nodes. Variety 'Sunbeam'. Duration of experiment 8 months. Eight replicates

Main shoot decapitated	Mean leaf number of lateral shoot to budding	Days to budding from date of decapitation	Height of lateral shoot at budding
(1) Immediately after end of vernalization . . .	20·0	85·4	18·9
(2) After production of 15 new leaves	23·1	73·5	16·4
(3) Immediately after budding	25·6	72·0	15·5
(4) After first appearance of petals	18·6	38·8	11·9
(5) After production of open flowers	9·0	25·3	8·5
S.E. (single mean) . .	±1·75	±3·98	±1·56

The possibility of de-vernalization of *lateral* buds also was further investigated, since this first experiment did not appear conclusive. For this purpose plants were decapitated at only two different stages of development but at five different levels in the plant. The first decapitation was carried out 4 weeks after the end of vernalization and at the following five levels: (1) below the youngest expanding leaf, (2) below the last fully expanded leaf, (3), (4), and (5) two, four, and seven leaves respectively below the last fully expanded leaf. The second series of decapitation treatments was delayed until the plants had formed macroscopically visible inflorescence buds. Level 1 in this case was the first axil below the lowest axillary inflorescence bud; the other four levels below this were arranged in steps roughly corresponding to those of the earlier decapitation treatment. The results presented in Table III show that the de-vernalization effect becomes more pronounced as the lower nodes of the plants are approached. Those laterals which became budded after the later decapitation treatment initiated considerably fewer leaves but required a longer time compared with the earlier series. This again suggests downward transfer of the stimulus. It may be surmised that the lower shoots which failed to bud had not yet been reached by the stimulus at the time of decapitation and became (or remained) de-vernalized.

TABLE III

*Effect of time and level of decapitation on vernalization status of axillary shoots. Variety 'Sunbeam'. Six replicates. DK 'A' = decapitated 4 weeks after end of vernalization, DK 'B' = decapitated after budding**

Level of decapitation in basi-petal order	Number of plants with budded laterals		Days to budding after decapitation		Leaf number on axillary shoot to budding		Leaf number on vegetative axillary at end of expt.	
	DK 'A'	DK 'B'	DK 'A'	DK 'B'	DK 'A'	DK 'B'	DK 'A'	DK 'B'
(1) Below youngest expanding leaf . .	6	6	19·0	68·7	12·1	7·3	..	..
(2) Below last fully expanded leaf . .	6	4	20·9	65·5	13·5	7·8	..	24·5
(3) Two leaves below level (2) . .	6	3	30·0	71·3	17·5	8·8	..	19·0
(4) Four leaves below level (2) . . .	6	4	38·7	70·5	21·1	9·0	..	16·0
(5 Seven leaves below level (2) . . .	4	0	46·5	>130	24·0	..	44·0	20·3

* Prior to decapitation the main shoots of these plants had budded 48·0 days after the end of vernalization with a mean leaf number increment of 25·7 leaves. In this treatment level (1) represents the first axil below the lowest axillary inflorescence bud; the other levels correspond to those of DK 'A' in their distances apart.

The results of these three experiments indicate that in natural de-vernalization internal factors such as the relative degrees of development of basal, axillary and main shoots, their distances apart, &c. may play a role in determining which shoots will become de-vernalized; and this aspect needs further clarification. But for the main point at issue clear evidence has been obtained, (1) that lateral and basal as well as terminal meristems are *initially*—at the end of cold treatment—fully vernalized, and (2) that the large majority of the basal shoots arising after the end of flowering of the main shoot are no longer vernalized. Hence, the annual need for vernalization is due to annual de-vernalization of at least the basal nodes on the main shoot.

An attempt was then made to discover whether this de-vernalization was merely an after-effect of the prolonged period of inactivity of these meristems during the growth and flowering of the main shoot. For this purpose young plants were sprayed with maleic hydrazide solutions. It is known that the development of plants can be arrested for considerable periods by this substance without actually killing them and that eventually normal growth may be resumed (Naylor and Davis, 1950). Young vernalized and unvernalized plants were sprayed over the entire foliage on several occasions with the following concentrations: 200, 1,000, and 5,000 p.p.m. The solutions were made up in a buffer at pH 6·0 and kept in a refrigerator (Naylor, loc. cit.); one drop of wetting agent was added immediately before use.

In the medium and higher concentrations growth was arrested almost completely for a considerable time after treatment. The young leaves failed to expand and remained yellowish in colour and had a rough, rather puckered surface. The older, mature leaves tended to become much more fleshy in appearance, perhaps as a consequence of starch accumulation (Greulach, 1953); those of the unvernalized plants were dark bluish green, but those of the vernalized set were noticeably paler, particularly round the veins. Both the higher concentrations, 1,000 p.p.m. and 5,000 p.p.m., caused the death of nearly all terminal growing points, but the plants remained alive and put out lateral shoots when growth was finally resumed. There was also some slight delay in the growth of those plants sprayed with 200 p.p.m., but as will be seen from Table IV their vernalization status remained unaffected. At the medium concentration no growth occurred for a period of nearly 5 weeks, after which time laterals from the axils of the older leaves started to grow out. Again their behaviour indicated no change in the vernalization status of the plants; all but one of the vernalized plants flowered, while all unvernalized plants remained so. The resumption of growth of the plants sprayed with 5,000 p.p.m. was delayed for over 12 weeks, when laterals began to develop from the lowest nodes. With the exception of two originally vernalized plants which failed to flower and one unvernalized plant which budded, there was again no change in the flowering behaviour of these plants thus prevented from growing for a period of 3 months. This period equals or even exceeds that of the growth of vernalized plants under similar conditions whose main shoots are allowed to flower normally. It would seem therefore that the period of inactivity of the basal shoots in itself is not likely to be the cause of their de-vernalization.

TABLE IV

Effect of maleic hydrazide (MH) spray on vernalization status. Variety 'Sunbeam'. Duration of experiment 150 days. Eight replicates

	Vernalized			Unvernalized			S.E. of mean
MH concentration p.p.m.	200	1,000	5,000	200	1,000	5,000	
Number of plants budded	8	7	6	..	..	1	..
Days to budding from:							
End of vernalization	45·1	66·4	127·3	..	..	121	±6·03
Mean date of resumption of growth	..	38·4	39·3	..	..	33	..
Leaf number to budding*	24·0	19·9	20·8	..	..	16·0	±1·5
Leaf number of vegetative plants*	..	37	19·5	43·8	27·5	33·1	
Mean internode length† (at budding)	0·80	0·57	0·36	0·21	0·21	0·26	±0·06

* Where the main growing point died, the mean value of all laterals was used.
† In vegetative plants measured only on mature part of stem.

Effects of high temperature treatment. In an attempt to investigate this de-vernalization further a number of experiments were carried out in which freshly vernalized cuttings were subjected to high temperature treatments similar to those known to be effective with winter rye and the biennial

Hyoscyamus niger. In the first experiment the following treatments were combined: two periods of vernalization (2 weeks and 4 weeks) and three periods of heat treatment (control, 4 hours heating and 30 hours heating). During the heat treatment one set of plants was kept in the light, the other in darkness, the two vernalization sets being treated on different occasions. As further control 3 unvernalized replicates were given the 0- and 30-hour heat treatments, the latter both in the light and in the dark. In each of the twelve main treatments six replicates were included. During the heat treatment the plants, grown in 3-in. pots, were supported immediately above the surface of a large water bath covered over by a glass sheet. In order to maintain a reasonably high light intensity sufficient for photosynthesis in those plants in the light two 1,000 W. lamps were suspended above the bath, giving about 500 ft. c. at plant level. Extra CO_2 was supplied to give about 1 per cent. CO_2 in the air which was stirred intermittently. The temperature was 40° C., 5° C. in excess of that known to be effective in de-vernalizing rye (Purvis and Gregory, 1952), and *Hyoscyamus* (Lang and Melchers, 1947). In the present experiment the temperature fluctuated slightly but was maintained within the limits of $40° \pm 2°$ after an initial period while the plants and soil were warming up. During the heat treatment of the plants vernalized for 4 weeks a temperature of 44° C. was touched for a brief moment on one occasion. The longest period of heat treatment, i.e. 30 hours, was chosen since marked de-vernalization had been found in the winter rye experiments with even shorter times, and also because technical difficulties prevented this temperature being maintained for such long periods as were used by Lang and Melchers (20 days).

Since the results of this experiment were negative a brief description of the effects will suffice. All plants survived the heat treatment, though 40° C. is probably close to the thermal death point of the Chrysanthemum. In a few plants given the 4 weeks vernalization treatment and heated for 30 hours in the dark, the terminal apices died, but a single lateral was allowed to grow and behaved identically with the remainder. In one treatment, however, all the replicate plants were killed outright, these were the unvernalized controls (not included in the factorial design) which were subjected to 30 hours heat in the dark—an observation which would suggest that perhaps the vernalization treatment actually makes the plants also more heat-resistant. Subsequently all heat-treated plants, especially the 30 hours set, showed a strong but temporary mottle of the leaves. The times to flowering and leaf-number data are shown in Table V. The only effect statistically highly significant in both characteristics is that between the two periods of vernalization. A slight but significant effect on time to flowering is also found between the three heating treatments; it appears to be due to somewhat earlier flowering in the set heated for 4 hours compared with the other two. A slight overall difference between the light and dark series is probably due to chance. The effects on internode lengths are also confined to the difference between the two periods of vernalization.

This failure to obtain 'heat de-vernalization' after only two weeks cold treatment suggested that in the Chrysanthemum 'fixation' of the vernalization

TABLE V

Effect of heat treatment at 40° C. for different times after two periods of vernalization. The heat treatment was given in the light and dark. Variety 'Sunbeam'. Six replicates. Extra unvernalized treatments, three replicates

	Vernalized for:	Duration of heat treatment (hrs.)							Sig. diffce. for main vern. effect
		0		4		30			
		Light	Dark	Light	Dark	Light	Dark	Mean	
Leaf-number increment to budding	2 weeks	27·7	26·1	29·1	27·1	29·0	27·3	27·8	0·76
	4 weeks	22·7	23·3	23·0	23·5	22·5	21·5	22·8	
	unvern.	40·7	..	..	..	44·7	..	42·7	..
Days to budding from end of vernalization	2 weeks	106·3	102·1	98·8	97·1	102·5	98·8	100·9	6·98
	4 weeks	82·1	81·3	79·7	81·7	91·0	84·1	83·3	
	unvern.	146·3	..	..	..	145·7	..	146·0	..
Mean internode lengths (cm.)	2 weeks	0·68	0·71	0·69	0·66	0·72	0·70	0·69	0·122
	4 weeks	0·81	0·77	0·85	0·79	0·76	0·91	0·82	
	unvern.	0·52	..	..	..	0·49	..	0·51	..

stimulus may take place even earlier than in rye and *Hyoscyamus* (a possibility later disproved entirely). For this reason an even shorter period of vernalization preceding heat treatment was used in another experiment. In this experiment three periods of vernalization (4, 8, and 12 days) were combined with three durations of heat treatment at 40° C. (0, 8, and 30 hours). To each of the nine treatments eight replicate plants were allotted, additional unvernalized controls being also run in the 0- and 30-hour heat treatments. All other conditions were similar to those in the previous test except that no attempt was made to give additional illumination during treatment and no extra CO_2 was supplied. The result of this experiment also was entirely negative and no significant de-vernalization was achieved. Final leaf-number data, &c. are shown in Table VI.

TABLE VI

Effect of heating at 40° C. on partly vernalized plants. Variety 'Sunbeam'. Six replicates for treatments vernalized for 0 days, all others eight replicates

	Vernalized for:	Duration of heat treatment (hrs.).		
		0	8	30
Number of plants budded	0 days	0	..	0
	4 days	0	0	0
	8 days	2	2	1
	12 days	7	7	5
Days to budding (excl. vegetative plants)	8 days	128·0	121·0	156·0
	12 days	107·4	97·1	76·2
Leaf number to budding (those of vegetative plants in brackets)	0 days	(57·8)	..	(57·5)
	4 days	(59·3)	(60·6)	(57·9)
	8 days	54·5 (63·5)	38·5 (61·1)	67 (60·3) No sig.
	12 days	51·6 (58)	46·9 (57)	35·0 (60·7) diffces.

From the results of these two experiments then it must be concluded that heat treatment for 30 hours immediately after vernalization, at as high a temperature as 40° C., cannot annul even the slight vernalization effect of 8–12 days cold treatment.

Finally an experiment was set up to investigate the effect of high-temperature treatment extended over a much longer period. On this occasion the plants to be de-vernalized were kept in a small glazed chamber in a greenhouse receiving 8 hours full daylight and 16 hours darkness under a light tight cloth. The air in the chamber was stirred continuously to eliminate temperature gradients and a high humidity was maintained. Two periods of vernalization were given, 14 days and 28 days; the heat treatment (35° C.) was given for 0, 10, 20, and 30 days. Eight replicate plants were used per treatment. The results are shown in Table VII. Again, all plants initiated inflorescences regardless of heat treatment, but inspection of the times required for budding to take place after the end of vernalization and also of leaf number increments would suggest that prolonged heating did after all lead to some de-vernalization. When, however, the number of days of such heat treatment is deducted from the total time these differences vanish. The same may be seen from the leaf-number increment subsequent to the end of heat treatment, which equals that of the unheated controls. Unfortunately this was determined only for the two 30-day heat treatments, but if the average increase in leaf number to budding over the controls is calculated for all treatments per 10-day period of heat treatment, the mean plastochrone is found to be about 2·8 days which corresponds closely to the normal rate of leaf initiation of one leaf every three days. It would appear therefore that no true de-vernalization had been achieved even by this prolonged heat treatment; and surprisingly the plants behaved as if they had retained through this period the flowering status they held at its beginning, normal progress towards flowering being resumed when they were returned to normal temperatures.

TABLE VII

Effect of prolonged heating at 35° C. for different times after two periods of vernalization. Variety 'Sunbeam'. Eight replicates

	Duration of Vernalization (days)								
	14				28				
Duration of heat treatment (days)	0	10	20	30	0	10	20	30	S.E. of mean
Number of plants budded . . .	8	8	8	8	8	8	8	8	..
Days to budding after end of vernalization .	32·4	36·4	47·5	54·6	27·1	36·8	45·8	54·4	±1·7
Days to budding after end of heat treatment	32·4	26·4	27·5	24·6	27·1	26·8	25·8	24·4	
Leaf-number increment to budding . .	16·4	19·4	23·5	26·0	14·1	19·6	25·0	25·8	±2·7
Leaf-number increment after end of heat treatment . . .	..	..	..	16·5	..	..	..	16·0	..

The prolonged heat treatment caused some changes in the morphology of the young leaves. Those which expanded at high temperature but had been initiated earlier failed to develop any stipules, while leaves initiated during heating possessed almost smooth margins with much-reduced notching.

De-vernalization by reduced light intensity. A chance observation then suggested a further method by which complete de-vernalization might possibly be obtained. Prolonged exposure to reduced light intensity came under suspicion as the cause of failure of some vernalized plants to flower, and a first experiment was set up to test this possibility. In this experiment only two treatments were given: normal vernalization (5 weeks cold) followed (1) by return to full light in the greenhouse (controls) or (2) by a period of 3 weeks with low intensity incandescent light (about 25 ft. c.) before return to normal daylight. A short day of 8 hours was maintained throughout. The results, shown in Table VIII, Experiment A, indicate that a significant delay in flowering had in fact occurred, but this was no longer than the time actually spent in reduced light; and de-vernalization, if indeed it had occurred at all, was not complete. In a second experiment, in which the same conditions were given but the reduced light period was extended to 4 weeks, a high degree of de-vernalization was achieved, the delay in budding, even after deducting the period in reduced light, being nearly 8 weeks; also the leaf-number increments from the end of cold treatment were almost doubled (Table VIII, Experiment B). Complete de-vernalization was achieved in a third experiment in which 30 days of reduced light were given (Table VIII, Experiment C); only 1 of the 15 replicates had budded at all when the experiment was ended after nearly 7 months. Some completely de-vernalized plants from this experiment are shown in Plate I (Figs. 1 and 2) side by side with the controls. In the

TABLE VIII

Effect of reduced light intensity treatment on fully vernalized plants. Variety 'Sunbeam'

	Expt.	Controls	Reduced light	't' calculated	Value of 't' required for probability level shown
Number of replicates	A	9	9	..	..
	B	12	12	..	..
	C	15	15	..	..
Period in reduced light (days)	A	..	21	..	..
	B	..	28	..	..
	C	..	30	..	..
Number of plants budded	A	9	9	..	..
	B	12	12	..	..
	C	15	1	..	..
Days to budding after vernalization	A	80·6	98·6	4·43	0·05 = 2·12
	B	85·7	167·6	23·8	0·01 = 3·85
	C	46·1	82	..	..
Leaf-number increment to budding (vegetative plants in brackets)	A	21·0	25·6	2·58	0·05 = 2·12
	B	22·2	41·8	13·2	0·01 = 3·58
	C*	31·9	35 (70·4)	22·5	0·001 = 3·69

* Total leaf numbers were recorded in experiment C.

de-vernalized plants relatively long internodes can be distinguished near the base of the stem (due to the initial vernalization) followed by very short internodes after de-vernalization. Also the diageotropic growth habit found in unvernalized short-day treated plants (Schwabe, 1950) appears clearly in these two figures. However, in such de-vernalized plants the 90° bend does not take place near the soil level as in ordinary unvernalized plants in short day, but in the region of the first few nodes formed after de-vernalization. It is suspected that there may also be an important interaction with temperature and it is proposed to investigate this aspect further in the future.

The effect of such reduced-light treatment following different periods of vernalization was explored in another experiment combining four periods of vernalization (1, 2, 3, and 4 weeks) with four periods of reduced light (0, 10, 20, and 30 days). The experiment was replicated ten times. As an additional control a few unvernalized plants were again subjected to the longest low-light treatment. The results are presented in Table IX.

TABLE IX

Effect of different lengths of reduced light intensity treatment on plants vernalized for different periods. Variety 'Sunbeam'. Ten replicates. (Unvernalized controls, six replicates)

	Vernalization period (days)	Duration of reduced light treatment (days)			
		0	10	20	30
Number of plants budded terminally	0	0	..	..	0
	7	0	0	0	0
	14	5	0	0	0
	21	10	3	0	0
	28	10	3	0	0
Number of plants with bud-laterals and vegetative terminal growing point	0	0	..	..	0
	7	0	0	0	0
	14	4	3	0	0
	21	..	5	2	0
	28	..	4	1	0
Days to budding	0	∞	..	..	∞
	7	∞	∞	∞	∞
	14	78·4	∞	∞	∞
	21	51·3	67·5	∞	∞
	28	41·3	67·0	∞	∞
Leaf number to budding (vegetative plants in brackets)	0	(61·1)	..	..	(53·5)
	7	(58·2)	(54·8)	(55·2)	(51·3)
	14	32·0 (60·0)	(63·3)	(56·0)	(51·6)
	21	25·8	38·3 (62·1)	(59·5)	(52·9)
	28	24·3	28·7 (59·7)	(57·1)	(52·9)

None of the plants vernalized for 1 week produced a bud and their flowering behaviour remained unaffected by the low-light treatment, as also was that of the unvernalized controls. With 2 weeks cold half the full-light controls became budded though with high leaf numbers; but here even 10 days of low-light treatment caused complete de-vernalization. With 3 or 4 weeks of cold the full vernalization effect was attained in the controls, but here too complete de-vernalization followed 20 or more days of reduced light. With only 10 days of this treatment three plants of each of these two low-temperature sets reached budding though with increased leaf numbers.

According to earlier observations it is not uncommon for partially vernalized plants to produce lateral shoots some way below the terminal apex, and for these to develop inflorescence buds although the main shoot itself remains strictly vegetative. This was again observed in the present experiments and the number of plants bearing such axillary flowering shoots is also shown in the table. An example of this phenomenon is illustrated in Plate II, Fig. 3.

In a further experiment an attempt was made to determine the stage after which treatment by reduced light becomes ineffective and vernalization irreversible, since in rye and *Hyoscyamus* this is known to occur during the first few days after return from the cold to normal temperatures. For this purpose 4 weeks of low light intensity were given, beginning 0, 1, 2, 3, and 4 weeks after the end of the low-temperature treatment. Before and after the reduced-light treatment the plants were grown in normal greenhouse conditions. The results of this experiment are summarized in Table X, from which it is seen that more than 3 weeks' growth in the greenhouse may take place before the vernalization stimulus becomes irreversible. From other experiments it is known that at about this time the first anatomical changes in the terminal apex leading to receptacle formation are taking place, and it would appear possible that in the Chrysanthemum the vernalization stimulus becomes irreversibly 'fixed' only as a consequence of the actual anatomical changes themselves.

TABLE X

Effect of four weeks de-vernalization by reduced light following different periods of growth after the end of vernalization. Variety 'Sunbeam'. Six replicates

	Number of weeks after end of vernalization when reduced-light treatment was started					
	0	1	2	3	4	
Number of plants budded . .	0	0	0	0	5*	..
Days to budding	∞	∞	∞	∞	43·0	..
Leaf-number increment to budding (vegetative plants in brackets)	(45·5)	(44·0)	(43·7)	(47·1)	36·0	No sig. diffce.
Mean internode length of vernalized part of stem	0·61	0·51	0·54	0·40	(*a*) 0·89† (*b*) 1·42	±0·073
Ditto of de-vernalized part . .	0·24	0·25	0·27	0·32	..	
Mean node number where change-over to de-vernalization occurred	15·4	16·1	18·7	21·4	..	..

* One plant died.

† Mean internode lengths of flowering plants at (*a*) budding and (*b*) final height attained.

In this experiment mean internode lengths both on the lower, vernalized, part of the stem and on the upper, unvernalized, part were measured and are shown in Table X, data which give a striking illustration of the effect of de-vernalization on internode elongation. The average node number (counting from below upwards) at which the change-over occurred is also shown.

DISCUSSION

The plants of which the temperature requirements have been studied most intensively in recent years are the winter cereals, often called 'winter annuals', and the so-called 'true biennials' such as *Hyoscyamus niger*. In these plants it is the young seedling or the 1-year-old plant which normally requires to be vernalized. The unvernalized stage in the life cycle of these plants therefore begins with the production of the seed. In the Chrysanthemum a rather different situation obtains. Here, the whole plant is normally subjected to cold conditions during the winter and, as has been shown in the results described above, all meristems, lateral as well as terminal, are vernalized at this time. Here then the unvernalized condition can arise only as the result of subsequent de-vernalization and in this respect the Chrysanthemum resembles more those perennial plants which form dormant winter buds (such as deciduous trees or plants like *Stratiotes aloides*) than rye or henbane. It would not seem impossible, however, that even in these cases the production of seed itself involves a process akin to de-vernalization.

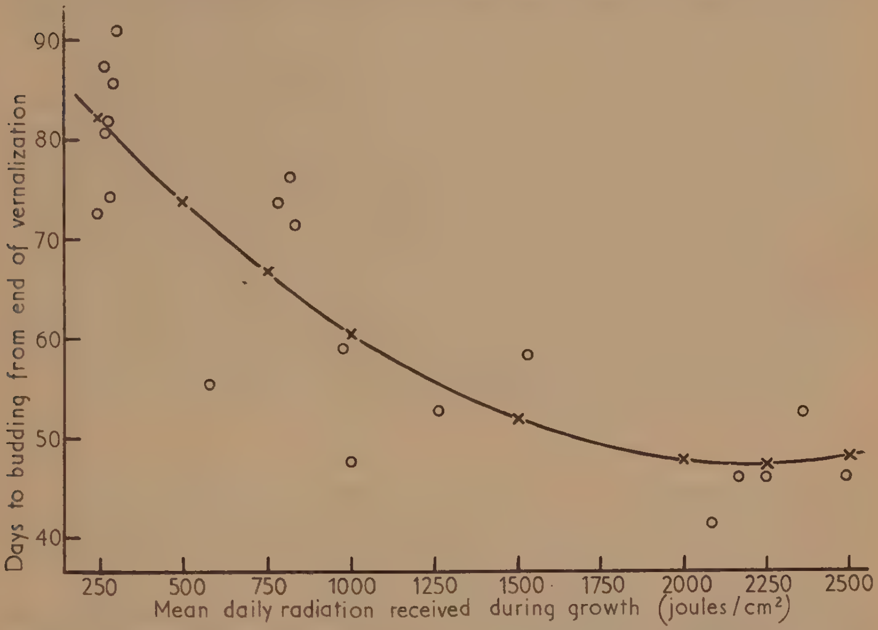
A further contrast appears in the reaction to heat treatment between the Chrysanthemum on the one hand and the rye and henbane plants on the other. The two last are easily de-vernalized provided the cold stimulus has not become fixed by prolonged low-temperature treatment or by allowing some time to elapse at normal temperatures immediately after vernalization. In the Chrysanthemum no true de-vernalization was obtained even by 30 days at 35° C. and no detectable effect at all by 40° C. for 30 hours. As was pointed out above, the delay in budding after, say, 30 days at high temperature corresponds surprisingly closely to 30 days; also the increase in total leaf number appears to be due only to those leaves produced during the heat treatment. Progress towards flowering seems therefore to be arrested completely during this period, but is then resumed at the same rate as in the un-heated controls, almost as if no heating had taken place. This contrasts sharply with the reduced light effects described above. Sen and Chakravarti (1946) failed to de-vernalize *Sinapis alba* also by heat treatment. It is probable, however, that temperature effects will prove to be important in interaction with the low-light treatment found to cause de-vernalization, and there may be a fairly high minimum temperature below which such de-vernalization treatment will not be fully effective.

Prolonged low-intensity light treatment will cause complete de-vernalization and also induce in the plants the same diageotropic growth habit which has been found in completely unvernalized plants grown in short day. This de-vernalization treatment has also been shown to be effective even after a considerable amount of growth has been made after return of the cold-treated plants to normal temperatures in the greenhouse. In fact the vernalization stimulus appears to remain labile until the stage has been reached when obvious morphological changes such as receptacle formation, &c. are about to begin. This would also appear to contrast somewhat with what occurs in the other two species cited. It may also be recalled here that even spring rye can be partly de-vernalized by anaerobic conditions at 20° C., and this variety must be fully 'vernalized'. Some preliminary experiments in which the reduced-light treatment was given to young plants (not sprouted seeds) of winter rye and to *Hyoscyamus* plants failed to induce any de-vernalization, though these results require confirmation. Of 40 rye seedlings vernalized as young plants, 20 were given 24 days of reduced-light treatment, but none of the 14 survivors failed to shoot. Although earing was delayed in these plants compared with the controls, their mean leaf number was actually reduced by more than one leaf. Similar negative results were obtained with *Hyoscyamus*.

No attempt has yet been made to investigate the effects of different intensities during the low-light treatment, but it would seem that for de-vernalization the illumination must be well below the compensation point. Even at a much higher level, light intensity has been shown to exert a very marked effect on the time to flowering, an effect which is quite distinct, however, from de-vernalization. The data were gathered from the control treatment of twenty different experiments carried out in the course of 4 years. In all these experiments fully vernalized plants of variety 'Sunbeam' were grown in an 8-hour day in a heated greenhouse whose temperature was kept reasonably constant throughout the year. Thus the only factor showing strong seasonable variation was light intensity. A regression of the mean times to budding (days) was calculated on the mean daily radiation received during the growth period. This was found to be highly significant and is shown in Text-fig. 4. The quadratic term of this regression was significant also, indicating that the approximately linear relation between light intensity and the time to flowering which prevails for a large part of the year ceases to hold in the high light intensities of the summer months. The effect of light intensity, however, probably acts indirectly via rate of photosynthesis on growth rate since the leaf-number increments to budding do not show such a correlation. Records of the total radiation measured by the Callendar recorder were obtained through the kindness of the Physics Department of Rothamsted Experimental Station, where these experiments were carried out.

The question whether the 'natural' de-vernalization is actually caused by low light intensity cannot yet be regarded as settled. There is no doubt that the young basal shoots usually arise from stolons growing below ground level and are likely to have been subject to low light conditions under the soil surface or in the shade of the mother plant. If the apical growing point of an unvernalized plant is made to grow in complete darkness the resulting shoot resembles an underground stolon in every respect and there is no doubt that these two organs do not essentially differ (Plate II, Fig. 5).

The temporary cessation of growth of the basal buds due to apical dominance of the main shoot may probably be excluded as the cause of their de-vernalization by the results obtained from the experiment with maleic hydrazide sprays. Also the 'taking' and re-rooting of such basal cuttings does not affect their vernalization status as has been shown earlier (Schwabe, 1952).



TEXT-FIG. 4. Relation between mean times to budding in short day and average light intensity received during period of growth. +——+ quadratic regression line.

What then is the mechanism of this reduced-light de-vernalization? Two possibilities suggest themselves between which it is hoped it may be possible to distinguish in future work. One possible explanation is carbohydrate starvation. Purvis (1947) has shown that sugar is needed for the vernalization of rye embryos and Claes (1947) also believes that carbohydrate level plays an important role in the flower induction in *Hyoscyamus niger*. If this be correct, one might further postulate that some factor formed during vernalization is actually broken down and lost in the course of what has been termed 'dissimilatory processes in the dark'—in this case at light intensities below compensation point.

The second possibility is that auxin effects are involved in de-vernalization. On the basis of the experiments described in the second paper of this series (Schwabe, 1951) it is clear also that changes in the auxin metabolism induced by light-intensity changes have a large effect on the inflorescence development of the Chrysanthemum. Numerous papers in the literature have described auxin effects on the flowering of plants. It is hoped that further experiments may allow a distinction to be made between these two possibilities, though, of course, it may be that both factors are involved simultaneously, or neither. One other factor which may be concerned is protein hydrolysis, for this is common to all the three known methods by which de-vernalization may be brought about (heat, anaerobiosis, and prolonged low-light-intensity treatment).

From an ecological point of view the occurrence of de-vernalization is likely to be of advantage to a perennial plant in preserving some viable shoots for the next season after the main axes have flowered—shoots which have short internodes and at least in some varieties show diageotropic growth even above soil level. They are thus more likely to escape frost damage either below the soil surface or even under a thin layer of snow. De-vernalization may also be the explanation of reports in practical horticulture of 'blindness' in the Chrysanthemum, which may be the result of long periods of low light intensity during the winter months.

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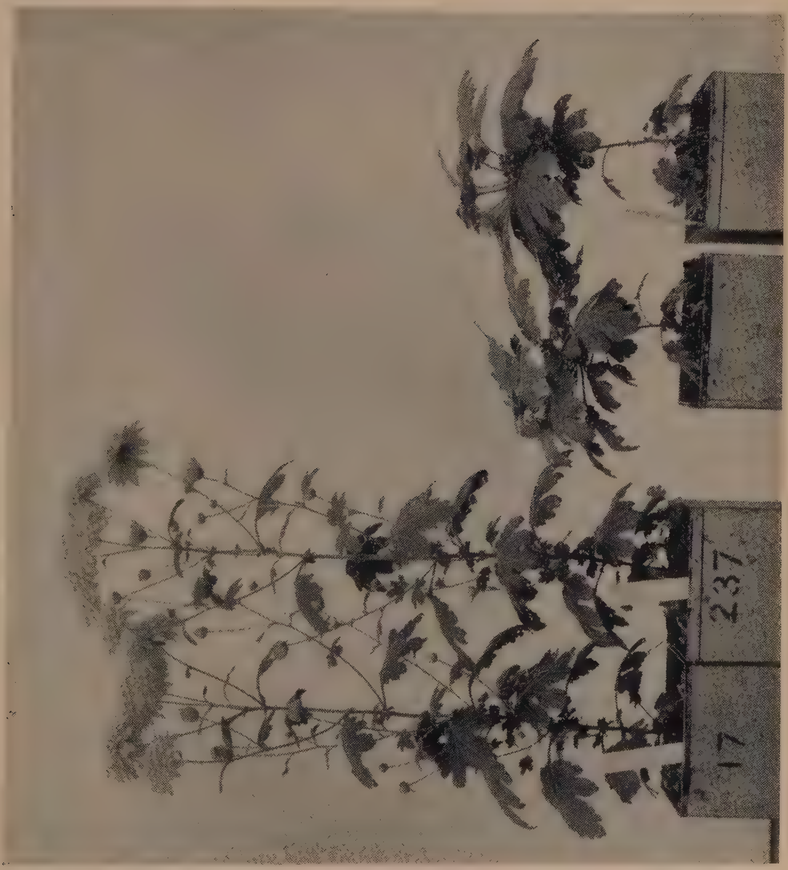

FIG. 1. De-vernalization caused by low intensity light treatment. Left controls, right de-vernalized plants. Note diageotropic growth of plant beginning above upright, vernalized part of stem.

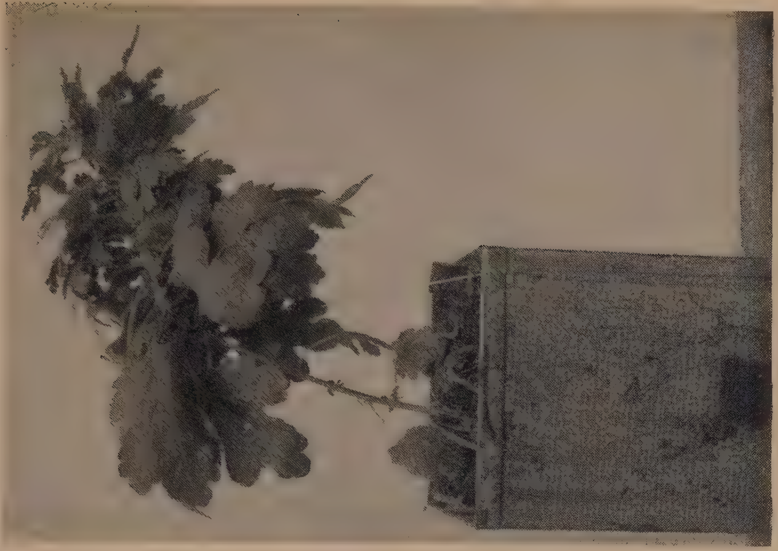

FIG. 2. De-vernalized plant at late stage of growth.

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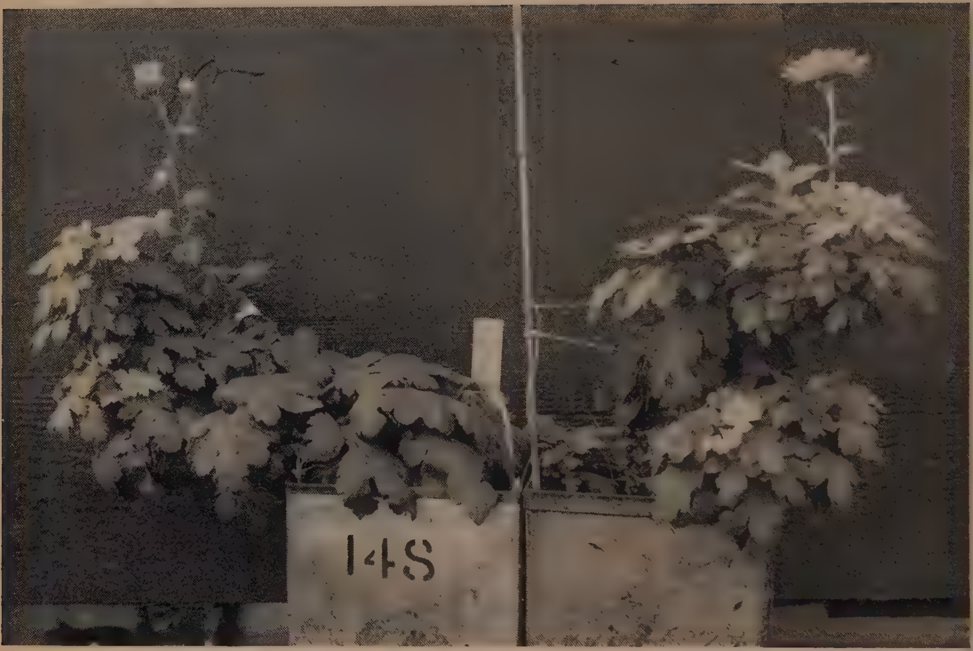

FIG. 3. Partly vernalized plants showing flowering axillary shoots on completely vegetative main shoot.

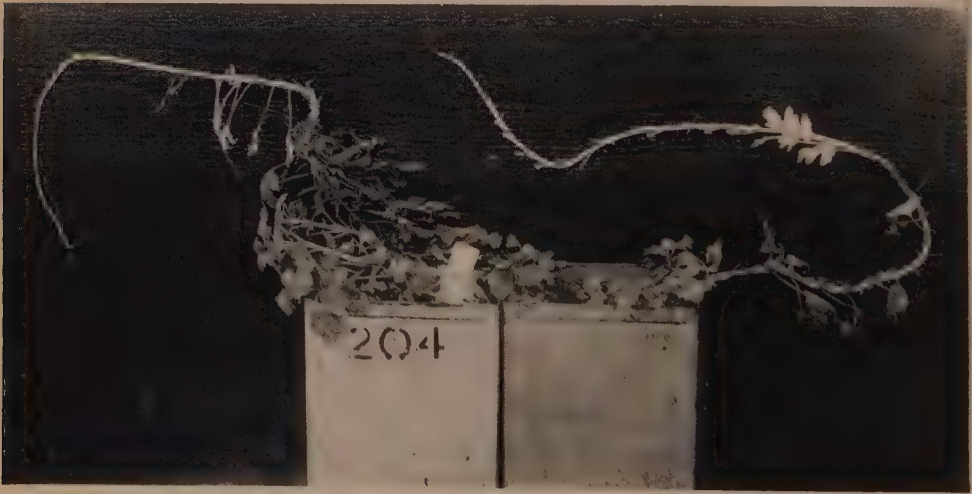

FIG. 5. Terminal shoots of unvernalized short-day treated plants grown in complete darkness showing entire similarity to underground stolons.

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A Biophysical Study of Etiolated Broad Bean Internodes

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WITH 7 PLATES

SUMMARY

The internodal cortical parenchyma of etiolated and normal broad bean seedlings has been examined by techniques appropriate to normal and polarized light, X-ray and electron diffraction, and the electron microscope. No fundamental differences in fine-structure were observed, the cellulose being in both cases organized into well-marked but only feebly crystalline microfibrils weakly oriented in a transverse direction. The cells produced in darkness, though larger and showing a reduced tendency to differentiate into collenchyma, are fundamentally normal in type; and the gross morphological differences must primarily be sought in a changed disposition of the products of cell-division at the apex.

INTRODUCTION

THE term 'etiolation' stands badly in need of redefinition. On its first recorded use (by Erasmus Darwin in 1791) and for at least a century afterwards it meant only the *blanching* of plants grown in darkness; and since no known angiosperm forms chlorophyll in darkness, it can be said that all known angiosperms etiolate. However, growth in darkness is frequently accompanied by morphological abnormalities, the remarkable variety of which is clearly demonstrated by MacDougal's (1903) monograph. Of these, the most spectacular—though by no means the commonest—is the excessive internodal elongation shown especially by the hypocotyls or epicotyls of certain seedlings. The striking nature of this phenomenon (in particular, its sensitivity to light exposures of very short duration) has naturally attracted much attention; and this has so obscured the original meaning of the word 'etiolation' that Miller (1931, p. 824) can write: 'When *a plant* is grown in darkness . . . it is greatly elongated, the tip of the stem has a characteristic hooked form, and the leaves remain very narrow and scalelike. A plant grown under such conditions *and with such an appearance* is said to be "etiolated" [our italics].' A distinct term is required for this by no means universal excessive growth in length, so that 'etiolation' may be confined to its original, and useful, meaning.

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We suggest the term *dolichosis* (δολιχός, long); our reasons for choosing this term from the several which suggest themselves are given in a note at the end of this paper.

Surprisingly little is known of the basic causes of dolichosis, it not even being known with certainty whether the increase is primarily in cell number or cell size. A number of parallel researches into this problem are in progress, or projected, in the Southampton laboratories; but we feel that the first essential is to ascertain whether the cells in dolichotic internodes are themselves normal, particularly in view of the differences in plasmolytic behaviour reported by Priestley (1926). It is conceivable, for example, that the walls contain an unusually low ratio of cellulose to other structural compounds, or that the cellulose chains are differently oriented. We wish to know, in fact, whether the apex is producing cells of a special type, or fundamentally normal cells in an unusual disposition. The biophysical techniques which have been developed in the Leeds laboratories are particularly appropriate to such an investigation.

MATERIAL

Plant material (*Vicia faba* L., the broad bean) was examined in the form of strips of internodal cortex about 3 cm. long, which were cut on a sledge microtome at thicknesses varying between 400 and 500 μ. Strips required for X-ray diffraction observations were allowed to dry in air on to clean glass microscope slides; others were fixed for 30 minutes in 5 per cent. aqueous formalin and stored in 50 per cent. alcohol. Any strips containing vascular tissue (usually leaf traces) were later discarded. Plants were grown either in continuous darkness for 10 to 15 days, or in light for about 3 weeks (before which time no internodes of adequate length are produced in light), so that the first dolichotic internode was being compared with the third or fourth normal internode; and several successive sowings were necessary to obtain enough material. Although all the plants were of the same horticultural variety (Toogood's Mammoth Green Longpod), the experimental material was thus far from homogeneous; however, we are seeking biophysical differences comparable in magnitude with the enormous difference in length between dolichotic and normal internodes, and we should expect that, if any such exist, they would transcend the inhomogeneity of our material.

EXPERIMENTAL RESULTS

1. *Direct microscopical examination.* Preserved strips were macerated for 3 days in 5 per cent. chromic acid in specimen tubes containing glass beads, which were shaken from time to time. The resulting material was centrifuged, washed, stained in Bismarck brown, mounted in 50 per cent. glycerine, and examined under the low power of the microscope. Photographs of groups of typical cells so obtained are shown in Pl. I, Figs. 1 and 2. The cells from dolichotic internodes are clearly larger, though the discrepancy in cell-length is of an altogether lower order than that in internode-length; the latter must,

therefore, be primarily due to a different rate of cell formation at the stem apex. There is, too, a marked difference in degree of differentiation. Many of the cells in light-grown internodes are more rigid, and more difficult to separate in macerating fluid; and many show a tendency to longitudinal thickened bands. It appears, in fact, that the cortex of light-grown internodes contains a high proportion of collenchyma.

2. *Polarized light*. Preserved strips were mounted in 50 per cent. glycerine and examined between crossed Nicols. Both light and dark strips proved to be weakly birefringent; and, surprisingly, the dark strips appeared at first sight to be the more so. Further examination, however, showed that there was again a difference due to a degree of differentiation. In the light strips the collenchyma was strongly birefringent but the parenchyma nearly isotropic, so that the appearance depended on the precise region in which the section was taken, and the consequent amount and distribution of collenchyma. The dark strips were more uniformly birefringent: less so than the collenchyma, but more so than the parenchyma, of the light strips. The long axis of the cells lies, as is to be expected, parallel to the long axis of the internode; if this is taken as the reference direction, examination with a colour-plate (Red I) shows that the birefringence is negative, the maximum extinction position being perpendicular to the long axis. The main orientation of the cellulose chains must therefore lie nearer to the transverse than to the longitudinal axis; as would be the case, for example, if they were laid down in a flat spiral.

Single cells, obtained by chromic acid maceration as described above, were now examined; a typical dark cell is shown in Pl. II, Fig. 3. The birefringence is nearly uniform, such slight banding as exists having been exaggerated by taking the photograph slightly off the position of maximum brightness. Note especially that the long axes of the pits are set almost transversely, which supports the suggestion that this is the main direction of cellulose orientation.

Ten such cells were examined under white light with a de Sénarmont compensator and gave a mean phase difference of –2·9° (SE. of mean 0·3°). Examination of a hand-section of fresh material under a 1/12 in. oil-immersion lens showed that the wall thickness was approximately 1 μ; if we take the wave-length of the light to be 0·5 μ, these measurements correspond to a wall birefringence of 0·004. However, the wall is not entirely cellulose. A sample consisting of 50 1-cm. lengths of dark-grown internode was crudely fractionated into 'pectin' (soluble in 0·5 per cent. ammonium oxalate), 'hemicellulose' (soluble in 4 per cent. NaOH) and 'cellulose' (insoluble in both); the method used was suggested to us by Dr. M. V. Tracey, and will be described in detail when current biochemical investigations have been completed. This analysis gave pectin 21·7 mg.; hemicellulose 10·9 mg.; cellulose 39·3 mg. (corresponding values for a similar sample of light-grown internode were pectin 29·5 mg.; hemicellulose 18·2 mg.; cellulose 50·8 mg.). The cellulose thus represents 55 per cent. of the structural material present; and if we assume that it alone is anisotropic, this gives us a birefringence for the cellulose of 0·007. For well-oriented chains at 16° to the transverse the value should be about 0·012

(Preston, 1952, p. 179); and since the pit-orientation suggests that the angle is almost certainly less than this, the value should be still higher. The low value must therefore represent either chains with a high angular dispersion (i.e. weak orientation), or a low crystalline/non-crystalline ratio, or both. The former alternative can be explored with the aid of the electron microscope; the latter, by X-ray or electron diffraction.

No comparable studies were carried out on light-grown cells; the parenchyma was too nearly isotropic for measurement with a de Sénarmont compensator, and the collenchyma too variable in birefringence for such results to have any meaning.

3. *X-ray diffraction.* X-ray diagrams were obtained using four superposed strips as specimen, the flat surface normal to Cu—K_α radiation collimated to a beam of 0·5 mm. diameter; exposure-times were between 20 and 30 hours. The results are shown in Pl. II, Figs. 4 and 5. Apart from salt rings, only the ring corresponding to the planes of 3·9 Å spacing appears, and this is in both cases a diffuse unbroken circle; there is no trace of arcs. The walls are therefore not highly crystalline, and orientation in the transverse direction cannot be precise.

It may be recalled that, under the conditions in which these photographs were taken, a series of flat spirals would give rise to a diagram consisting of complete circles with highest intensity along the meridian (Preston, 1946). This would naturally be smoothed out by any pronounced angular dispersion. The absence of circles corresponding to planes of spacing 5·4 Å and 6·1 Å recalls the condition in conifer cambium (Preston and Wardrop, 1949) and again would seem to indicate low crystallinity. The X-ray findings are therefore in harmony with the results of polarization microscopy.

4. *Electron microscope.* Preserved material was macerated in a blendor, and examined on 'Formvar' films after Pd—Au shadowing at $\tan^{-1} 1/3$. We may state immediately that we have been unable to discern any consistent differences between light- and dark-grown specimens, and the plates we reproduce have been selected to demonstrate particular structural features irrespective of the material from which they were obtained.

We were at first puzzled by the fact that some of the fragments appeared to be fibrous and others granular, a contradiction which is simply resolved by Pl. III, Figs. 6 and 7. The wall is two-layered: one of the layers is granular, with no trace of a fibrous structure, the other is markedly fibrous. The granular layer, which is on top in Fig. 6 and underneath in Fig. 7, we believe to be the middle lamella; this interpretation is supported by the fact that it has not been observed in material subjected to prior maceration in chromic acid. Such maceration, moreover, clarifies the fibrous structure, as is shown by Pl. IV, Fig. 8. There is an undeniable, but only weakly defined, orientation; the angular dispersion is very high, and there is a suggestion of a second direction of orientation, with fewer microfibrils, at an angle of about 50° to the first. The degree of orientation varied considerably between different fragments.

The records are not quite sharp enough to enable us to measure the microfibrils accurately, but approximate measurements show that the finest visible have a width of about 100 Å, coarser microfibrils (2 or 3 times this width) also being present. 'Islands of synthesis' (Preston and Kuyper, 1951; Preston, Nicolai, and Kuyper, 1953; Preston and Ripley, 1954*b*) are present on the inner surface of the wall, as is clearly shown by Pl. IV, Fig. 9.

It remains to determine the relationship between the major axis of microfibril orientation and the long axis of the entire cell, and here we have been extremely fortunate. One grid bore a very large piece of material which remained rigidly in position after the 'Formvar' film had, inevitably, broken; from its shape and size it appeared to represent not less than a quarter of a cell, and may even have been an entire small cell. Its orientation could be established beyond reasonable doubt, both the longitudinal thickened bands and the almost transverse pits being clearly visible. Part of it is shown in Pl. V; the white streak in the top right-hand corner represents (from comparisons with photographs taken at lower magnifications) the long axis of the intact cell. It is immediately clear that the main axis of microfibril-orientation, though it varies somewhat from one region to another, lies roughly transversely. This is striking confirmation of the deductions made from the observations in polarized light; and we believe this to be the first case in which an axis of orientation, established under the electron microscope, has been correlated with the long axis of the entire cell.

This specimen has also thrown considerable light on the structure of the pits (more strictly, pit-fields), one of which is shown in Pl. VI. The pit itself is almost circular; the elongated appearance under crossed Nicols is evidently due to a breakdown of orientation either side of the pit, as the fibres diverge to skirt it and then converge once more. The pit is not merely a thin place in the wall; it is a definite and circumscribed structure, traversed by a thin fibrous membrane pierced by apertures that we take to represent plasmodesmata. The structure is even more clearly demonstrated by the elliptical pit shown in Pl. VII, in which the pit-closing membrane has become detached and is seen slightly displaced to one side.

5. *Electron diffraction.* The electron microscope was set up for electron diffraction observations as described by Preston and Ripley (1954*a*). It was not even possible to distinguish definite rings, and there was no trace of arcs. There therefore seems no doubt that the material, though fibrous, is only very feebly crystalline, a situation already recorded for certain algae (Nicolai and Preston, unpublished).

DISCUSSION

We have found no fundamental differences in fine-structure between the cells of dolichotic and normal internodes. The biochemical data given above (p. 453) show that the proportions of the major structural constituents differ by only 3 per cent., which is probably no greater than the accuracy of the method. In both cases cellulose is organized into well-marked but only feebly crystalline microfibrils weakly oriented in a nearly transverse direction, and so probably constituting an irregular flat spiral. Our observations throw no light on the difference in plasmolytic behaviour reported by Priestley (1926); but any such difference is likely to reside in the structure of the innermost boundary of the cellulose wall, which is inevitably damaged by the relatively crude method of fixation here adopted. This region may be accessible by the use of protoplasmic fixatives followed by ultra-thin sectioning and electron microscope observation; we hope to investigate this possibility on a future occasion, and meanwhile the plasmolytic phenomena will themselves be re-investigated.

Morphological differences must clearly be sought in the number, size, distribution, or degree of differentiation of the cells, or in some combination of these. Opinions differ as to whether the rate of cell-division is increased or decreased by the action of light. Avery, Burkholder, and Creighton (1937), working with the coleoptile and first internode of *Avena* seedlings, find as we do that the differences in cell-length are inadequate to explain the differences in organ-length, and conclude that light inhibits cell-division. Parkinson (1950), however, working with pea seedlings, finds that in light there are more leaves and more cells per leaf; he therefore concludes that light stimulates cell-division. Whether or no there is any change in the rate of cell-division—and, in the absence of a study of *all* the products of apical division, neither of these investigations is (or claims to be) conclusive—there is no doubt that the *disposition* of the cells is changed. In light a high proportion of the cells are involved in the formation of leaf-primordia, whereas in darkness the great majority are diverted to the axis. This 'redistribution' of growth activity was pointed out by Priestley and Ewing (1923), though Priestley's (1926) suggestion that it can be attributed to the formation of endodermis in darkness has been shown by Bond (1935) to be untenable. Our own observations further suggest that in darkness extension growth of the cells of the internodal cortex continues for a longer period and is associated with a slower rate of differentiation into collenchyma.

It is clear that a complete cytological and anatomical study of the development of the apex is absolutely essential for further progress; and it is hoped to undertake this in due course.

A NOTE ON THE TERM 'DOLICHOSIS'

Words derived from *tendere* are inadmissible because of their implication of a process of stretching. Of the available roots meaning simply lengthening, *mekynsis* is unfamiliar and *macrosis* ambiguous; we therefore prefer *dolichosis*. Appropriate prefixes would be *hyper-* (implying that the elongation is excessive) or *scoto-* (implying that it takes place in darkness). The most accurate term would undoubtedly be *scotodolichosis*; but, in view of its ungainliness, we prefer to use *dolichosis*, leaving the longer form in reserve for any occasion on which greater refinement of meaning is desirable.

ACKNOWLEDGEMENTS

We are much indebted to Mr. W. E. Middleton, who prepared the cortical strips; and to Mr. M. D. Macleod for etymological advice.

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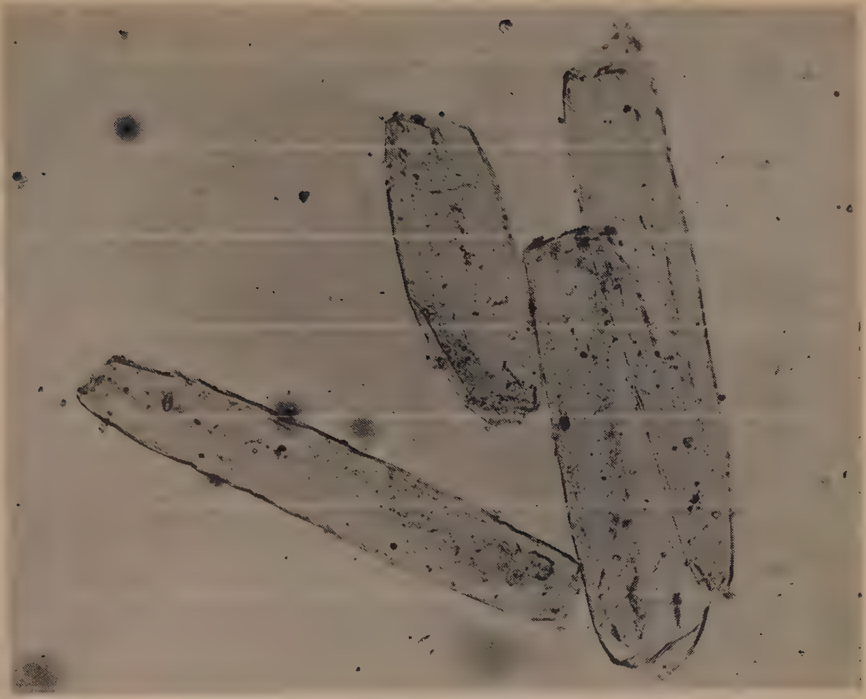

FIG. 1

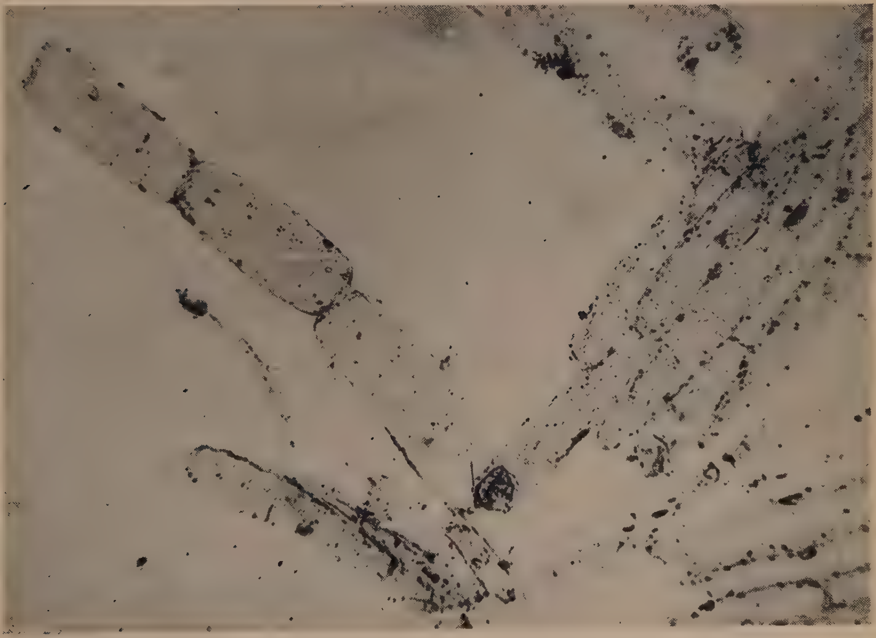

FIG. 2

FIG. 1. Cells from dark-grown internode. FIG. 2. Cells from light-grown internode. White lines represent $100\,\mu$ intervals

W. T. WILLIAMS, R. D. PRESTON, *and* G. W. RIPLEY—PLATE I

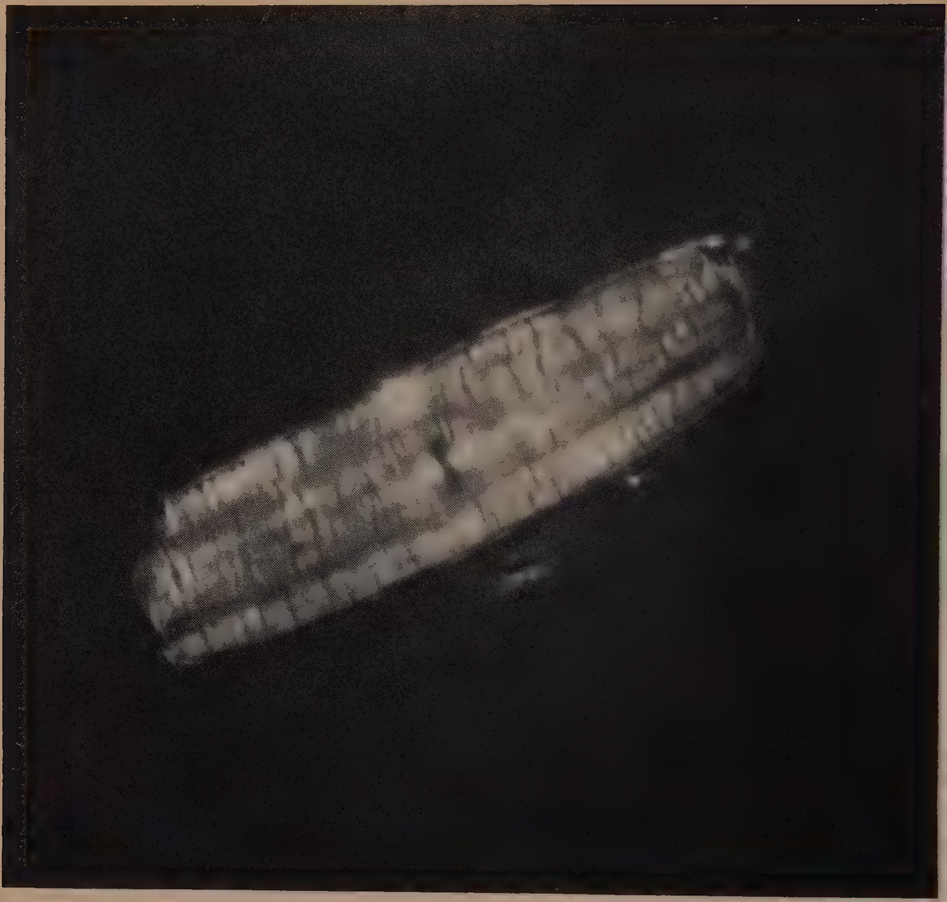

FIG. 3

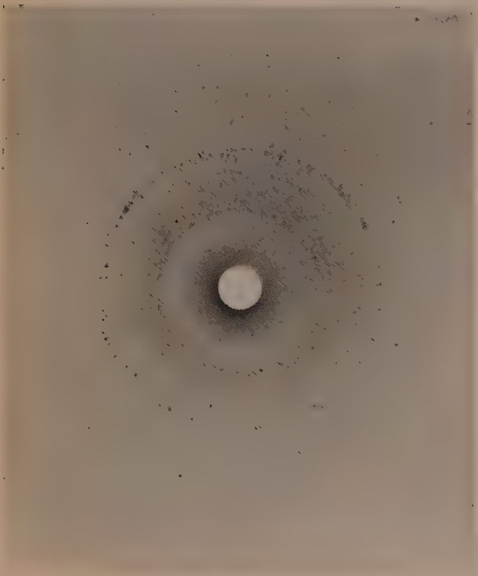

FIG. 4

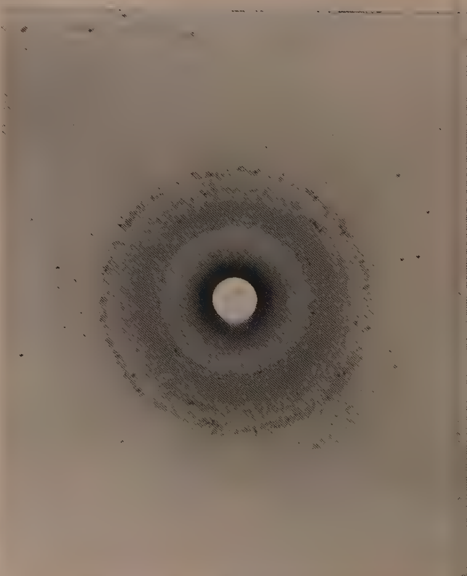

FIG. 5

FIG. 3. Cell (approx. 520 μ long) from dark-grown internode, photographed under crossed Nicols
FIG. 4. X-ray diffraction diagram, dark-grown internodal strips. FIG. 5. X-ray diffraction diagram

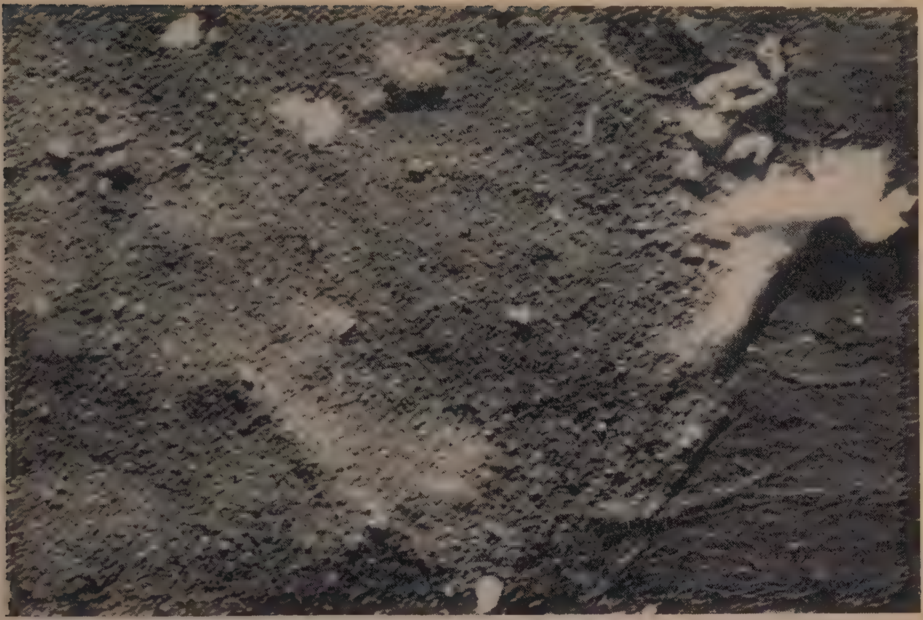

FIG. 6. Electron micrograph, dark-grown internode. × 30,000

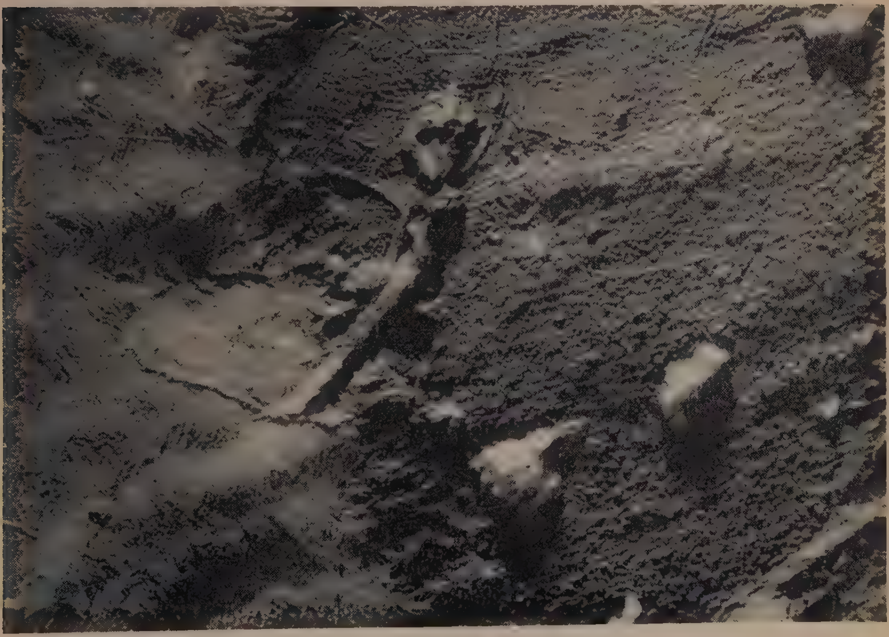

FIG. 7. Electron micrograph, dark-grown internode. × 30,000

W. T. WILLIAMS, R. D. PRESTON, *and* G. W. RIPLEY—PLATE III

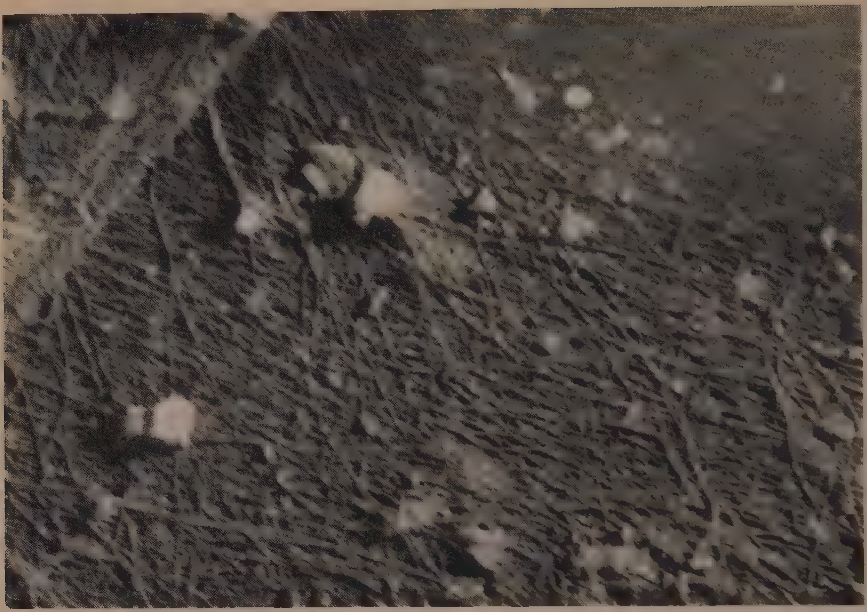

FIG. 8. Electron micrograph, light-grown internode, H_2CrO_4 maceration. × 30,000

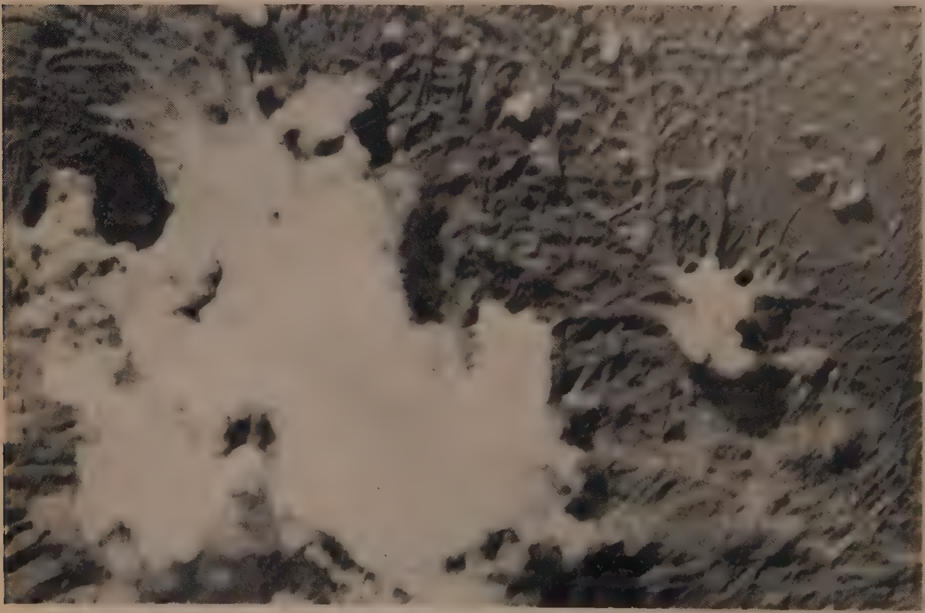

FIG. 9. Electron micrograph, light-grown internode, H_2CrO_4 maceration. × 30,000

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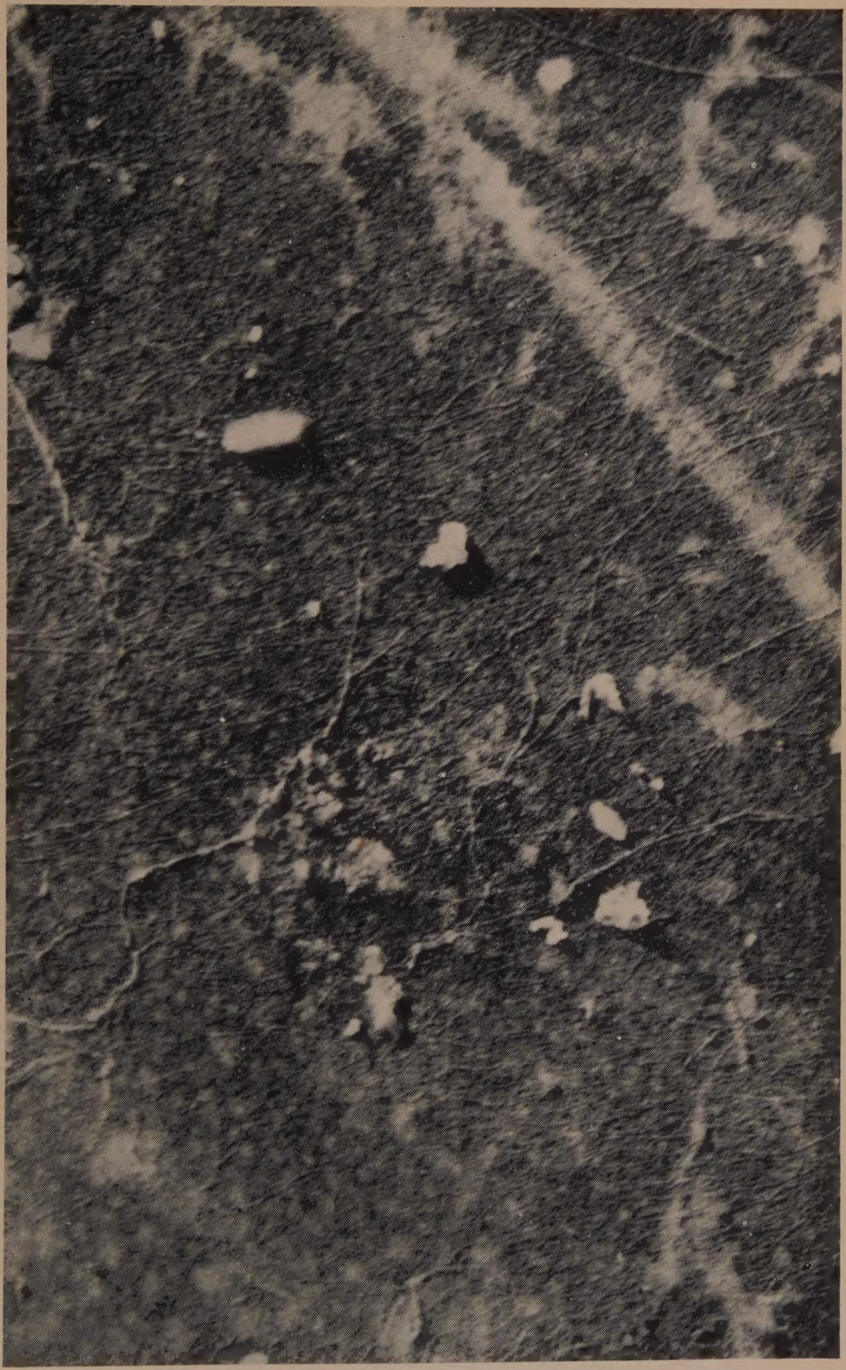
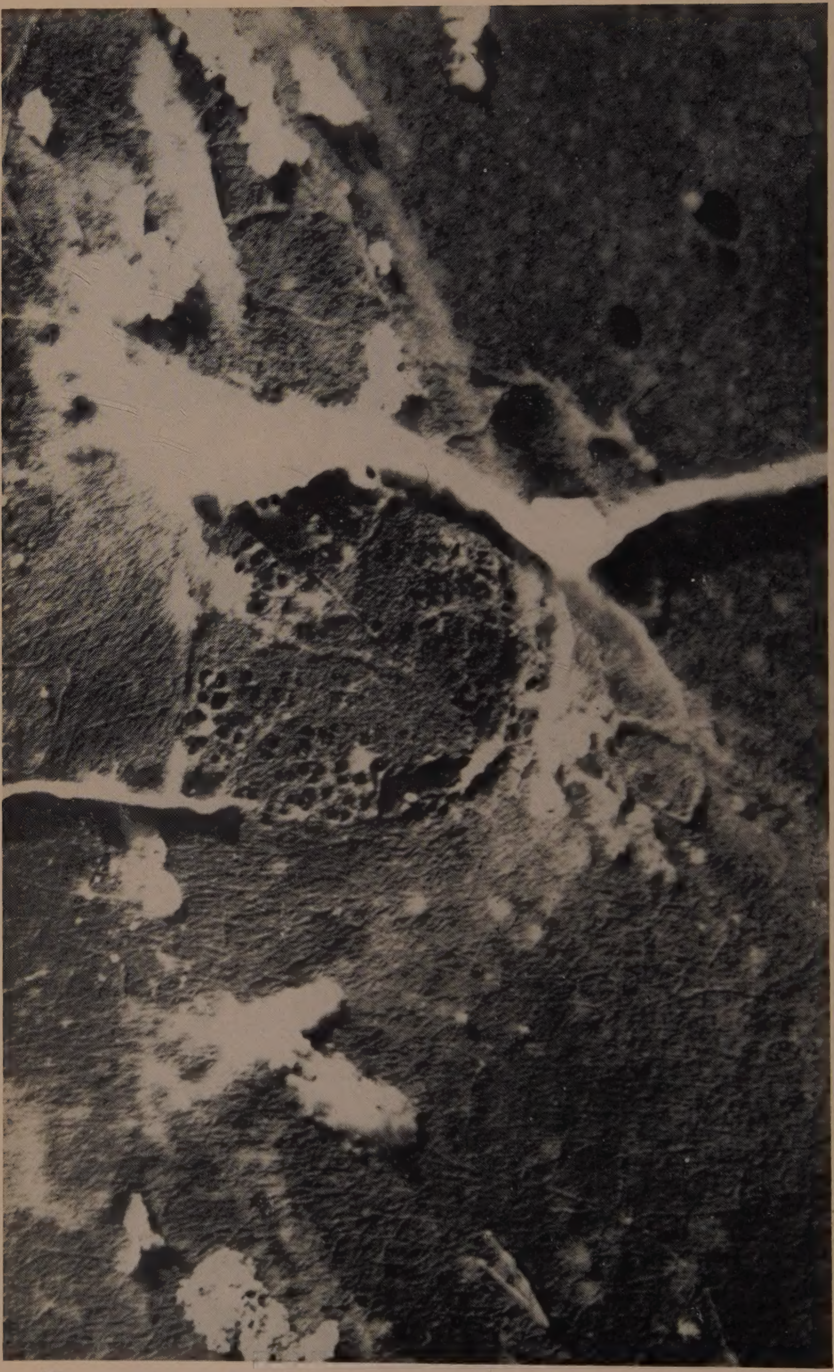


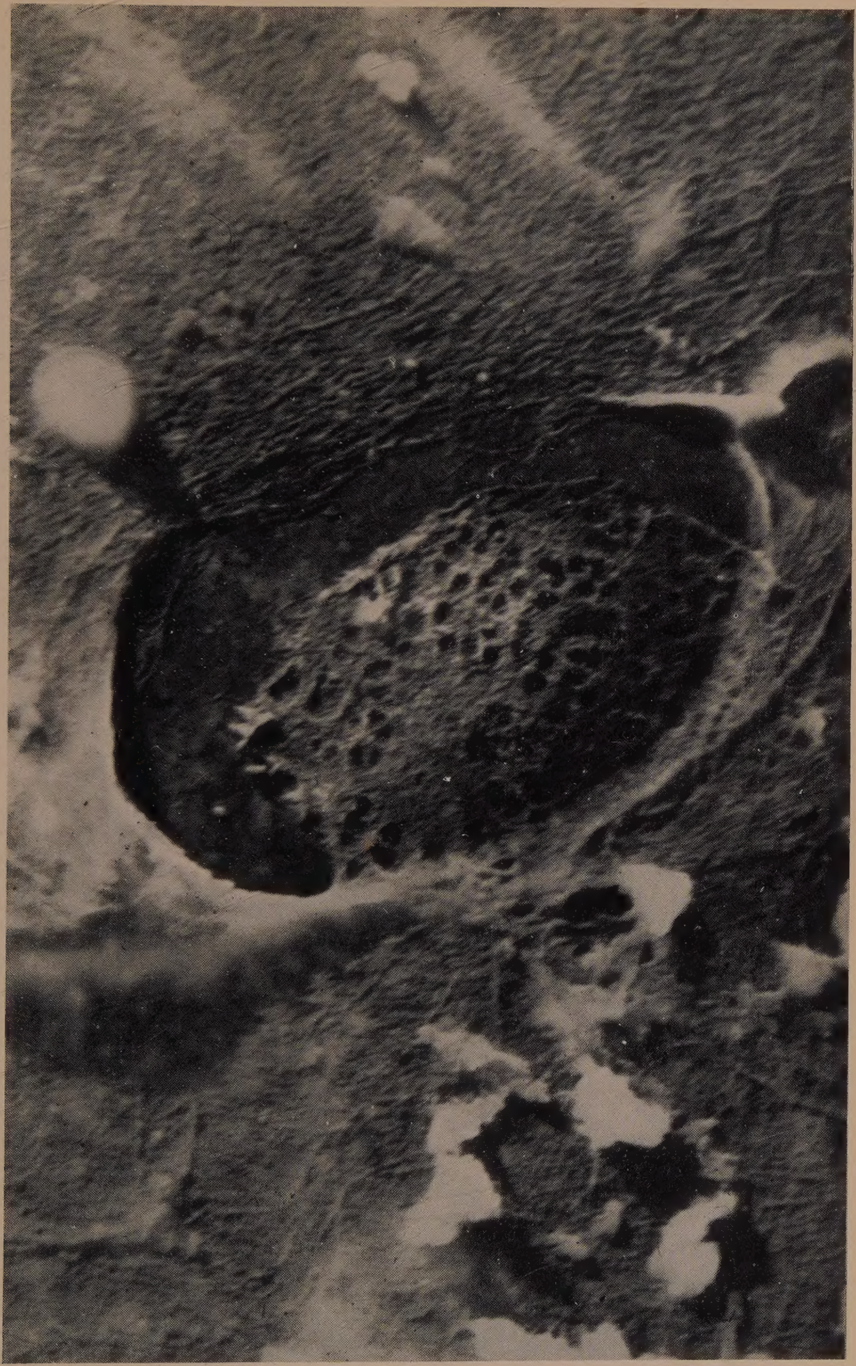
Electron micrograph, dark-grown internode, H_2CrO_4 maceration. × 15,000

W. T. WILLIAMS, R. D. PRESTON, *and* G. W. RIPLEY—PLATE V



Electron micrograph, dark-grown internode, H_2CrO_4 maceration. × 15,000

W. T. WILLIAMS, R. D. PRESTON, and G. W. RIPLEY—PLATE VI



Electron micrograph, dark-grown internode, H_2CrO_4 maceration. ×22,500

W. T. WILLIAMS, R. D. PRESTON, *and* G. W. RIPLEY—PLATE VII